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(54) Title: RECEPTOR BASED ANTAGONISTS AND METHODS OF MAKING AND USING

(57) Abstract

The present invention provides a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex. It also provides a nucleic acid sequence encoding the fusion polypeptide and methods of making and uses for the fusion polypeptide.

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RECEPTOR BASED ANTAGONISTS AND METHODS OF MAKING AND USING

This application claims priority of U.S. Application No. 09/313,942, filed May 19, 1999, which claims priority of U.S. Provisional Application No. 60/101,858 filed September 25, 1998. Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

BACKGROUND OF THE INVENTION

Although discovered for varying biological activities, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM) and interleukin-6 (IL-6) comprise a defined family of cytokines (referred to herein as the "CNTF family" of cytokines). These cytokines are grouped together because of their distant structural similarities [Bazan, J. Neuron 7: 197-208 (1991); Rose and Bruce, Proc. Natl. Acad. Sci. USA 88: 8641-8645 (1991)], and, perhaps more importantly, because they share "β" signal-transducing receptor components [Baumann, et al., J. Biol. Chem. 265:19853-19862 (1993); Davis, et al., Science 260: 1805-1808 (1993); Gearing et al., Science 255:1434-1437 (1992); Ip et al., Cell 69: 1121-1132 (1992); Stahl, et al., J. Biol. Chem. 268: 7628-7631 (1993); Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Receptor activation by this family of cytokines results from either homo- or hetero-dimerization of these β components [Davis, et al. Science 260: 1805-1808 (1993), Murakami, et al., Science 260: 1808-1810 (1993); Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. IL-6 receptor activation requires homodimerization of gp130 [Murakami, et al. Science 260: 1808-1810 (1993), Hibi, et al., Cell 63: 1149-1157 (1990)], a protein initially identified as the IL-6 signal transducer [Hibi, et al., Cell 63: 1149-1157 (1990)]. CNTF, LIF and OSM receptor activation results from heterodimerization between gp130 and a second gp130-related protein known as LIFRβ [Davis,

et al., Science 260: 1805-1808 (1993)], that was initially identified by its ability to bind LIF [Gearing et al., EMBO J. 10: 2839-2848 (1991)].

In addition to the β components, some of these cytokines also require specificity-determining "α" components that are more limited in their tissue distribution than the β components, and thus determine the cellular targets of the particular cytokines [Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Thus, LIF and OSM are broadly acting factors that may only require the presence of gp130 and LIFRβ on responding cells, while CNTF requires CNTFRα [Stahl and Yancopoulos, Cell 74: 587-590 (1993)] and IL-6 requires IL-6Rα [Kishimoto, et al., Science 258: 593-597 (1992)]. Both CNTFRα (Davis et al., Science 259:1736-1739 (1993) and IL-6Rα [Hibi, et al. Cell 63:1149-1157, Murakami, et al., Science 260:1808-1810 (1990); Taga, et al., Cell 58:573-581 (1989)] can function as soluble proteins, consistent with the notion that they do not interact with intracellular signaling molecules but that they serve to help their ligands interact with the appropriate signal transducing β subunits [Stahl and Yancopoulos, Cell 74: 587-590 (1993)].

Additional evidence from other cytokine systems also supports the notion that dimerization provides a common mechanism by which all cytokine receptors initiate signal transduction. Growth hormone (GH) serves as perhaps the best example in this regard. Crystallographic studies have revealed that each GH molecule contains two distinct receptor binding sites, both of which are recognized by the same binding domain in the receptor, allowing a single molecule of GH to engage two receptor molecules [de Vos, et al., Science 255: 306-312 (1992)]. Dimerization occurs sequentially, with site 1 on the GH first binding to one receptor molecule, followed by the binding of site 2 to a second receptor molecule [Fuh, et al., Science 256: 1677-1680 (1992)]. Studies with the erythropoietin (EPO) receptor are also consistent with the importance of dimerization in receptor activation, as EPO receptors can be constitutively activated by a

single amino acid change that introduces a cysteine residue and results in disulfide-linked homodimers [Watowich, et al., Proc. Natl. Acad. Sci. USA 89:2140-2144 (1992)].

In addition to homo- or hetero-dimerization of β subunits as the critical step for receptor activation, a second important feature is that formation of the final receptor complex by the CNTF family of cytokines occurs through a mechanism whereby the ligand successively binds to receptor components in an ordered manner [Davis, et al. Science 260:1805-1818 (1993); Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Thus CNTF first binds to CNTFRα, forming a complex which then binds gp130 to form an intermediate (called here the αβ1 intermediate) that is not signaling competent because it has only a single β component, before finally recruiting LIFRβ to form a heterodimer of β components which then initiates signal transduction. Although a similar intermediate containing IL-6 bound to IL-6Rα and a single molecule of gp130 has not been directly isolated, we have postulated that it does exist by analogy to its distant relative, CNTF, as well as the fact that the final active IL-6 receptor complex recruits two gp130 monomers. Altogether, these findings led to a proposal for the structure of a generic cytokine receptor complex (Figure 1) in which each cytokine can have up to 3 receptor binding sites: a site that binds to an optional α specificity-determining component (α site), a site that binds to the first β signal-transducing component (β1 site), and a site that binds to the second β signal-transducing component (β2 site) [Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. These 3 sites are used in sequential fashion, with the last step in complex formation -- resulting in β component dimerization -- critical for initiating signal transduction [Davis, et al. Science 260:1805-1818 (1993)]. Knowledge of the details of receptor activation and the existence of the non-functional β1 intermediate for CNTF has led to the finding that CNTF is a high affinity

antagonist for IL-6 under certain circumstances, and provides the strategic basis for designing ligand or receptor-based antagonists for the CNTF family of cytokines as detailed below.

Once cytokine binding induces receptor complex formation, the dimerization of β components activates intracellular tyrosine kinase activity that results in phosphorylation of a wide variety of substrates [Ip, et al. Cell 69:121-1132 (1992)]. This activation of tyrosine kinase appears to be critical for downstream events since inhibitors that block the tyrosine phosphorylations also prevent later events such as gene inductions [Ip, et al., Cell 69:121-1132 (1992); Nakajima and Wall, Mol. Cell. Biol. 11:1409-1418 (1991)]. Recently, we have demonstrated that a newly discovered family of non-receptor tyrosine kinases that includes Jak1, Jak2, and Tyk2 (referred to as the Jak/Tyk kinases) [Firmbach-Kraft, et al., Oncogene 5:1329-1336 (1990); Wilks, et al., Mol. Cell. Biol. 11: 2057-2065 (1991] and that are involved in signal transduction with other cytokines [Argetsinger, et al., Cell 74:237-244 (1993); Silvennoinen, et al., Proc. Natl. Acad. Sci. USA 90:8429-8433 (1993); Velazquez, et al., Cell 70: 313-322 (1992); Witthuhn, et al., Cell 74:227-236 (1993)], preassociate with the cytoplasmic domains of the β subunits gp130 and LIFRβ in the absence of ligand, and become tyrosine phosphorylated and activated upon ligand addition [Stahl et al., Science 263:92-95 (1994)]. Therefore these kinases appear to be the most proximal step of intracellular signal transduction activated inside the cell as a result of ligand binding outside of the cell. Assay systems for screening collections of small molecules for specific agonist or antagonist activities based on this system are described below.

The CNTF family of cytokines play important roles in a wide variety of physiological processes that provide potential therapeutic applications for both antagonists and agonists.

SUMMARY OF THE INVENTION

An object of the present invention is the production of cytokine antagonists that are useful in the treatment of cytokine-related diseases or disorders.

Another object of the invention is the use of the disclosed cytokine antagonists for the treatment of cytokine-related diseases or disorders. For example, an IL-6 antagonist described herein may be used for the treatment of osteoporosis, the primary and second effects of cancers, including multiple myeloma, or cachexia.

Another object of the invention is the development of screening systems useful for identifying novel agonists and antagonists of cytokine receptors.

Another object of the invention is the development of screening systems useful for identifying small molecules that act as agonists or antagonists of the cytokines.

Another object of the invention is the development of screening systems useful for identifying novel agonists and antagonists of members of the CNTF family of cytokines.

Another object of the invention is the development of screening systems useful for identifying small molecules that act as agonists or antagonists of the CNTF family of cytokines.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: Ordered binding of receptor components in a model of a generic cytokine receptor. The model indicates that cytokines contain up to 3 receptor binding sites and interact with their receptor components by

binding first the optional α component, followed by binding to β1, and then β2. The β components for many cytokine receptors interact through membrane proximal regions (shaded boxes) with the Jak/Tyk family of cytoplasmic protein tyrosine kinases. Only upon dimerization of β components is signal transduction initiated, as schematized by the tyrosine phosphorylations (P) of the β components and the Jak/Tyk kinases.

FIGURE 2: CNTF inhibits IL-6 responses in a PC12 cell line (called PC12D) that expresses IL6Rα, gp130, CNTFRα , but not LIFRβ. Serum-deprived PC12D cells were incubated + IL-6 (50 ng/mL) in the presence or absence of CNTF as indicated. Some plates also received soluble IL6Rα (1 mg/mL) or soluble CNTFRα (1 mg/mL) as indicated. Cell lysates were subjected to immunoprecipitation with anti-gp130 and immunoblotted with anti-phosphotyrosine. Tyrosine phosphorylation of gp130 is indicative of IL-6 induced activation of the IL-6 receptor system, which is blocked upon coaddition of CNTF.

FIGURE 3: Scatchard analysis of iodinated CNTF binding on PC12D cells. PC12D cells were incubated with various concentrations of iodinated CNTF in the presence or absence of excess non-radioactive competitor to determine the specific binding. The figure shows a Scatchard plot of the amount of iodinated CNTF specifically bound, and gives data consistent with two binding sites with dissociation constants of 9 pM and 3.4 nM.

FIGURE 4. The amino acid sequence of human gp130-Fc-His_6. Amino acids 1 to 619 are from human gp130 (Hibi et al., Cell 63:1149-1157 (1990). Note that amino acid number 2 has been changed from a Leu to a Val in order to accommodate a Kozak sequence in the coding DNA sequence. The signal peptide of gp130-Fc-His_6 has been italicized (amino acids 1 to 22). The Ser-Gly bridge is shown in bold type (amino acids 620, 621). Amino acids 662 to 853 are from the Fc domain of human IgG1 (Lewis, et

al., J. Immunol. 151:2829-2838 (1993). (†) mark the two cysteines (amino acids number 632 and 635) of the IgG hinge preceding the Fc that form the inter-chain disulfide bridges that link two Fc domains. The hexahistine tag is shown in bold/italic type (amino acids 854 to 859). (•) shows the position of the STOP codon.

FIGURE 5: The amino acid sequence of human IL-6Rα-Fc. Key: Amino acids 1 to 358 are from human IL-6Rα (Yamasaki, et al., Science 241:825-828 (1988). Note that amino acid number 2 has been changed from a Leu to a Val in order to accommodate a Kozak sequence in the coding DNA sequence. The signal peptide of IL-6Rα-Fc has been italicized (amino acids 1 to 19). The Ala-Gly bridge is shown in bold type (amino acids 359, 360). Amino acids 361 to 592 are from the Fc domain of human IgG1 (Lewis et al., J. Immunol. 151:2829-2838 (1993). (†) mark the two cysteines (amino acids number 371 and 374) of the IgG hinge preceding the Fc that form the inter-chain disulfide bridges that link two Fc domains. (•) shows the position of the STOP codon.

FIGURE 6: The CNTF/IL-6/IL-11 receptor system. The ordered formation of the hexameric signal transducing receptor complex is depicted schematically. The cytokine associates with the Rα component to form an obligatory cytokine•Rα complex (Kd is about 5 nM). This low affinity complex next associates with the first signal transducing component, marked β1, to form a high affinity cytokine•Rα•β1 complex (Kd is about 10 pM). In the case of IL-6Rα, this component is gp130. This trimeric high affinity complex subsequently associates with another such complex. Formation of this complex results in signal transduction as it involves dimerization of two signal transducing components, marked β1 and β2 respectively (adapted from (Ward et al., J. Bio. Chem. 269:23286-23289 (1994); Stahl and Yancopoulos, J. Neurobiology 25:1454-1466 (1994); Stahl and Yancopoulos, Cell 74:587-590 (1993).

FIGURE 7: Design of heterodimeric receptor-based ligand traps for IL-6. The heterodimeric ligand trap is comprised of two interdisulfide linked proteins, gp130-Fc and IL-6Rα-Fc. The gp130-Fc•IL-6Rα-Fc complex (upper panel) is shown to mimic the high affinity cytokine•Rα•β1 complex (lower panel). The ligand trap functions as an antagonist by sequestering IL-6 and thus rendering unavailable to interact with the native receptors on IL-6-responsive cells.

FIGURE 8. Heteromeric immunoglobulin Heavy/Light Chain Receptor Fusions. An example of a heavy/light chain receptor fusion molecule is schematically depicted. The extracellular domain of gp130 is fused to Cγ, whereas the extracellular domain of IL-6Rα is fused to the constant region of the kappa chain (κ). The inter-chain disulfide bridges are also depicted (S-S).

FIGURE 9. Amino acid sequence of gp130-Cγ1. Key: Amino acids 1 to 619 are from human gp130 (Hibi, et al., Cell 63:1149-1157 (1990). Ser-Gly bridge is shown in bold type. Amino acids 662 to 651 are from the constant region of human IgG1 (Lewis et al., J. Immunol. 151:2829-2838 (1993). (*) shows the position of the STOP codon.

FIGURE 10: Amino acid sequence of gp130Δ3fibro. Key: Amino acids 1 to 330 are from human gp130 (Hibi et al., Cell 63:1149-1157 (1990). Other symbols as described in Figure 9.

FIGURE 11: Amino acid sequence of J-CH1. Key: The Ser-Gly bridge is shown in bold, the J-peptide is shown in italics, the C_H1 domain is underlined.

FIGURE 12: Amino acid sequence of Cγ4. Key: The Ser-Gly bridge is shown in bold type. Amino acids 2 to 239 comprise the Cγ4 sequence.

FIGURE 13: Amino acid sequence of κ-domain. Key: The Ser-Gly bridge is shown in bold type. Amino acids 2 to 108 comprise the κ domain. The C-terminal cysteine (amino acid 108) is that involved in the disulfide bond of the κ domain with the C_H1 domain of Cγ.

FIGURE 14: Amino acid sequence of λ-domain. Key: The Ser-Gly bridge is shown in bold type. Amino acids 2 to 106 comprise the λ domain (Cheung, et al., J. Virol. 66: 6714-6720 (1992). The C-terminal cysteine (amino acid 106) is that involved in the disulfide bond of the λ domain with the C_H1 domain of Cγ.

FIGURE 15: Amino acid sequence of the soluble IL-6Rα domain. Key: Amino acids 1 to 358 comprise the soluble IL-6Rα domain (Yamasaki, et al., Science 241:825-828 (1988). The Ala-Gly bridge is shown in bold type.

FIGURE 16: Amino acid sequence of the soluble IL-6Rα313 domain: Key: Amino acids 1 to 313 comprise the truncated IL-6Rα domain (IL-6Rα313). The Thr-Gly bridge is shown in bold type.

FIGURE 17: Purification of gp130-Cγ1•IL-6Rα-κ. 4% to 12% SDS-PAGE gradient gel run under non-reducing conditions. Proteins were visualized by staining with silver. Lane 1: approximately 100 ng of material purified over Protein A Sepharose (Pharmacia). Lane 2: Molecular size standards (Amersham). Lane 3: The Protein A-purified material shown here after further purification over an IL-6 affinity chromatography step. The positions of the gp130-Cγ1 dimer [$(gp130\text{-}C\gamma1)_2$], the gp130-Cγ1 dimer

associated with one IL-6Rα-κ [(gp130-Cγ1)$_2$•(IL-6Rα-κ)$_1$], and the gp130-Cγ1 dimer associated with two IL-6Rα-κ [(gp130-Cγ1)$_2$•(IL-6Rα-κ)$_2$] are shown, as well as the sizes for the molecular size standards in kilodaltons (200, 100, and 46).

FIGURE 18: IL-6 dissociates slowly from the ligand trap. The dissociation rate of IL-6 from a heavy/light chain receptor-based ligand trap (gp130-Cγ1•IL-6Rα-κ) was compared to that obtained with the neutralizing monoclonal antibody B-E8 (BE8 MAb).

FIGURE 19: IL-6 can induce multimerization of the ligand trap. (A) Two different ligand traps are depicted schematically and listed according to their ability to bind protein A. gp130-Fc•IL-6Rα-Fc (GF6F) binds protein A via its Fc-domains, whereas gp130-C$_H$1•IL-6Rα-κ (G16K) does not bind to protein A. (B) Anti-kappa western blotting of proteins precipitated with Protein A-Sepharose from mixtures of GF6F ± IL-6, G16K ± IL-6, or GF6F plus G16K ± IL-6, as marked.

FIGURE 20: Inhibition of IL-6-dependent XG-1 cell proliferation. XG-1 cells [Zhang, et al., Blood 83:3654-3663 (1994)] were prepared for a proliferation assay by starving the cells from IL-6 for 5 hours. Assays were set up in 96-well tissue culture dishes in RPMI + 10% fetal calf serum + penicillin/streptomycin + 0.050 nM 2-mercaptoethanol + glutamine. 0.1 ml of that media was used per well. Cells were suspended at a density of 250,000 per ml at the start of the assay. 72 hours post addition of IL-6 ± ligands traps or antibodies, an MTT assay was performed as described (Panayotatos et al. Biochemistry 33:5813-5818 (1994). The different ligand traps utilized are listed.

FIGURES 21A-21D: Nucleotide sequence encoding and deduced amino acid sequence of fusion polypeptide designated 424 which is capable of binding the cytokine IL-4 to form a nonfunctional complex.

FIGURES 22A-22D: Nucleotide sequence encoding and deduced amino acid sequence of fusion polypeptide designated 603 which is capable of binding the cytokine IL-4 to form a nonfunctional complex.

FIGURES 23A-23D: Nucleotide sequence encoding and deduced amino acid sequence of fusion polypeptide designated 622 which is capable of binding the cytokine IL-4 to form a nonfunctional complex.

FIGURE 24A-24F: Nucleotide sequence encoding and deduced amino acid sequence of fusion polypeptide designated 412 which is capable of binding the cytokine IL-6 to form a nonfunctional complex.

FIGURE 25A-25F: Nucleotide sequence encoding and deduced amino acid sequence of fusion polypeptide designated 616 which is capable of binding the cytokine IL-6 to form a nonfunctional complex.

FIGURE 26A-26E: Nucleotide sequence encoding and deduced amino acid sequence of fusion polypeptide designated 569 which is capable of binding the cytokine IL-1 to form a nonfunctional complex.

FIGURE 27: Shows that an IL-4 trap designated 4SC375, which is a fusion polypeptide of IL-2Rγ-scb-IL4Rα-FcΔC1, is several orders of magnitude better as an IL-4 antagonist than IL4RαFcΔC1 alone in the TF1 cell bioassay.

FIGURE 28: Shows that an IL-4 trap designated 4SC375 displays antagonistic activity in the TF1 cell bioassay equivalent to an IL-4 trap designated 4SC424 (described in Figs. 21A-21D) which is a fusion

polypeptide of IL-2Rγ-IL4Rα-FcΔC1 having the IL-2Rγ component flush with the IL-4Rα component.

FIGURE 29: Shows that the IL6 trap (6SC412 IL6R-scb-gpx-FcΔC1) described in Figs. 24A-24F is a better antagonist of IL-6 in the XG1 bioassay than the neutralizing monoclonal antibody to human IL-6 - BE8.

FIGURE 30: Shows that the trap 1SC569 (described in Figs. 26A-26E) is able to antagonize the effects of IL-1 and block the IL-6 production from MRC 5 cells upon treatment with IL-1.

FIGURE 31A-31G: The nucleotide and encoded amino acid sequence of the IL-4Rα.IL-13Rα1.Fc single chain trap construct is set forth.

FIGURE 32A-32G: The nucleotide and encoded amino acid sequence of the IL-13Rα1.IL-4Rα.Fc single chain trap construct is set forth.

FIGURE 33: Blocking of IL-13 by IL-4Rα.IL-13Rα1.Fc and IL-13Rα1.IL-4Rα.Fc. Addition of either IL-4Rα.IL-13Rα1.Fc or IL-13Rα1.IL-4Rα.Fc trap at a concentration of 10nM blocks IL-13-induced growth up to ~2nM. At an IL-13 concentration of ~4-5 nM the growth of TF1 cells is inhibited by 50%.

FIGURE 34: Blocking of IL-4 by IL-4Rα.IL-13Rα1.Fc and IL-13Rα1.IL-4Rα.Fc. Addition of either IL-4Rα.IL-13Rα1.Fc or IL-13Rα1.IL-4Rα.Fc at a concentration of 10nM blocks IL-4-induced growth up to ~1nM. At an IL-4 concentration of ~3-4 nM the growth of TF1 cells is inhibited by 50%.

FIGURE 35: Human IL-1 trap blocks the in vivo effects of exogenously administered huIL-1. BALB/c mice were given subcutaneous injection of huIL-1 (0.3 μg/kg) at time 0. Twenty-four hours prior to huIL-1 injection, the animals were pre-treated with either vehicle or 150-fold molar excess

of huIL-1 trap. Two hours prior to sacrifice (26 hrs), the mice were re-challenged with a second injection of huIL-1 (0.3 μg/kg, s.c.). Blood samples were collected at various time points and sera were assayed for IL-1 levels (expressed as mean +/- SEM; n=5 per group).

FIGURE 36A & FIGURE 36B: Human IL-4 trap antagonizes the effects of human IL-4 in monkeys. Figure 36A: Cynomologus monkeys were treated in three parts as indicated. Human IL-4 (25 μg/kg) was injected subcutaneously twice daily for 4 days and human IL-4 trap (8 mg/ml) and vehicle were given intravenously daily for 5 days, beginning 1 day prior to human IL-4 administration. Plasma was collected daily and assayed for MCP-1 levels. Results were expressed as mean +/- SEM; n=4. (ANOVA $p<0.0007$; Tukey-Kramer: Part 2 vs. Part 1, p,0.05; Part 2 vs. Part 3, p,0.05; Part 1 vs. Part 3, not significant.) Figure 36B: Cynomologus monkeys were treated in three parts as indicated. Human IL-4 (25 μg/kg) was injected subcutaneously twice daily for 4 days and human IL-4 trap (8 mg/ml) and vehicle were given intravenously daily for 5 days, beginning 1 day prior to human IL-4 administration. Whole blood was collected daily for flow cytometry analysis for CD16. Results were expressed as mean +/- SEM; n=4. (ANOVA $p<0.042$; Tukey-Kramer: Part 2 vs. Part 1, $p<0.05$; Part 2 vs. Part 3 and Part 1 vs. Part 3, not significant.)

FIGURE 37: Murine IL-4 trap partially prevented IL-4-mediated IgE increase in mice. BALB/C mice injected with anti-mouse IgD (100μl/mouse, s.c.) were randomly divided into 3 groups, each received (on days 3-5) either vehicle, murine IL-4 trap (1 mg/kg, s.c.), or a monoclonal antibody to mouse IL-4 (1 mg/kg, s.c.). Sera were collected at various time points and assayed for IgE levels. Results were expressed as mean+/-SEM (n=5 per group). (ANOVA $p=0.0002$; Tukey-Kramer: vehicle vs. IL-4 trap, $p<0.01$; vehicle vs. IL-4 antibody, $p<0.001$; IL-4 trap vs. IL-4 antibody, not significant).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex comprising:

a) a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the specificity determining component of the cytokine's receptor;

b) a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the signal transducing component of the cytokine's receptor; and

c) a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component.

By "cytokine binding portion" what is meant is the minimal portion of the extracellular domain necessary to bind the cytokine. It is accepted by those of skill in the art that a defining characteristic of a cytokine receptor is the presence of the two fibronectin-like domains that contain canonical cysteines and of the WSXWS box (Bazan, J.F., 1990, PNAS 87: 6934-6938). Sequences encoding the extracellular domains of the binding component of the cytokine's receptor and of the signal transducing component of the cytokine's receptor may also be used to create the fusion polypeptide of the invention. Similarly, longer sequences encoding larger portions of the components of the cytokine's receptor may be used. However, it is contemplated that fragments smaller than the extracellular domain will function to bind the cytokine and therefore, the invention contemplates fusion polypeptides comprising the minimal portion of the extracellular domain necessary to bind the cytokine as the cytokine binding portion.

The invention comprises a "specificity determining component" of a cytokine's receptor and a "signal transducing component" of the cytokine's receptor. Regardless of the nomenclature used to designate a particular component or subunit of a cytokine receptor, one skilled in the art would recognize which component or subunit of a receptor is responsible for determining the cellular target of the cytokine, and thus would know which component constitutes the "specificity determining component."

Similarly, regardless of the nomenclature used, one of skill in the art would know which component or subunit of a receptor would constitute the "signal transducing component." As used herein, the "signal transducing component" is a component of the native receptor which is not the specificity determining component and which does not bind or weakly binds the cytokine in the absence of the specificity determining component. In the native receptor, the "signal transducing component" may participate in signaling.

For example, while some cytokine receptors have components designated α and β, the IL-4 receptor has a signal transducing component referred to as IL-2Rγ. However, regardless of what name is associated with that component, one skilled in the art would know which component of the IL-4 receptor is the signal transducing component. Thus to practice the present invention and create a high affinity trap for IL-4, one of skill in the art would create an isolated nucleic acid comprising a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the specificity determining component of the IL-4 receptor (IL-4Rα); a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the signal transducing component of the IL-4 receptor (IL-2Rγ); and a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a

multimerizing component (for example, an Fc domain of IgG) to create a high affinity trap for IL-4.

Some further examples of the receptor components that may be used to prepare cytokine antagonists according to the invention are set forth in Table 1. The Table 1 sets forth, by way of example but not by way of limitation, some of the varied nomenclature used in the scientific literature to describe those components which function as specificity determining components and those which function as signal transducing components of certain cytokine receptors.

TABLE 1

Cytokine	Specificity determining Component	Signal transducing Component
Interleukin-1 (IL-1)	Type I IL-1R (ref. 8)	IL-1R AcP (refs. 8, 11)
	Type II IL-1R (ref. 8)	
	IL-1RI (ref. 11)	
	IL-1RII (ref. 11)	
Interleukin-2 (IL-2)	α-subunit (ref. 2)	β-chain (ref. 3)
	α-chain (ref. 3)	β-subunit (ref. 2)
	IL-2Rα (ref. 1)	γ-chain (ref. 3)
		IL-2Rβ (refs. 1, 10)
		IL-2Rγ (refs. 1, 10)
Interleukin-3 (IL-3)	IL-3Rα (ref. 1)	β_c (ref. 1)
	α-subunit (ref. 2)	β-subunit (ref. 2)
	α-receptor component (ref. 5)	β-chain (ref. 3)
		β-receptor component (ref. 5)
Interleukin-4 (IL-4)	IL-4R (ref. 1)	γ-chain (ref. 3)
		IL-2Rγ (ref. 1)
Interleukin-5 (IL-5)	IL-5Rα (ref. 1)	β_c (ref. 1)
	α-subunit (ref. 2)	β-subunit (ref. 2)
	α-receptor component (ref. 5)	β-chain (ref. 3)
		β-receptor component (ref. 5)

TABLE 1 (CONT'D)

Cytokine	Specificity determining Component	Signal transducing Component
Granulocyte macrophage-colony stimulating factor (GM-CSF)	α-receptor component (ref. 5) α-subunit (ref. 2) GMRα (refs. 1, 2)	β-receptor component (ref. 5) β-subunit (ref. 2) β-chain (ref. 3) β_c (ref. 1) GMRβ (refs. 1, 2)
Leukemia inhibitory factor (LIF)	LIFBP (ref. 1) α-receptor component (ref. 5)	gp130 (refs. 1, 3) β- receptor component (ref. 5)
Interleukin-11 (IL-11)	α–chain (ref. 4) NR1 (ref. 4)	gp130 (ref. 4)
Interleukin-15 (IL-15)	IL-15Rα (ref. 10)	IL-2Rβ (ref. 10) IL-2Rγ (ref. 10)
Interferon-γ (IFNγ)	IFN-γR (ref. 7) IFN-γR1 (ref. 7)	AF-1 (ref. 7) IFN-γR2 (ref. 7)
TGFβ	Type II (refs. 6, 9)	Type I (refs. 6, 9)

Only a few of the multitude of references are cited in Table 1, and they are set forth as follows:

1. Sato and Miyajima, Current Opinions in Cell Biology 6: 174-179 (1994) - See page 176, lines 9-16;

2. Miyajima, et al., Annual Review of Immunology 10: 295-331 (1992) - See page 295, line 4 to page 296, line 1; page 305, last paragraph;

3. Kondo, et al, Science 262: 1874-1877 (1993) - See page 1874, cols. 1 & 2;

4. Hilton, et al, EMBO Journal 13: 4765-4775 (1994) - See page 4766, col. 1, lines 20 24;

5. Stahl and Yancopoulos, Cell 74: 587-590 (1993) - See page 587, column 2, lines 15-22;

6. Bassing, et al, Journal of Biological Chemistry 269: 14861-14864 (1994) - See page 14861, col. 2, lines 1-9 and 21-28;

7. Kotenko, et al, Journal of Biological Science 270: 20915-20921 (1995) - See page 20915, lines 1-5 of the abstract;

8. Greenfeder, et al., Journal of Biological Chemistry 270: 13757-13765 (1995) - See page 13757, col. 1, line 6 to col. 2, line 3 and col. 2, lines 10-12; page 13764, col. 2, last 3 lines and page 13765, col. 1, lines 1-7;

9. Lebrun and Vale, Molecular Cell Biology 17: 1682-1691 (1997) - See page 1682, Abstract lines 2-6;

10. Kennedy and Park, Journal of Clinical Immunology 16: 134-143 (1996) - See page 134, lines 1-7 of the abstract; page 136, col 2., lines 1-5;

11. Wesche, et al., Journal of Biological Chemistry 272: 7727-7731 (1997) See page 7731, lines 20-26.

Kotenko, et al. recently identified the IL-10R2 (IL-10Rβ) chain which is reported to serve as an accessory chain that is essential for the active IL-10 receptor complex and for initiating IL-10 induced signal transduction events (S.V. Kotenko, et al., The EMBO Journal, 1997, Vol. 16: 5894-5903). Additional cytokines and their receptors are described in Appendix II, page A:9 of Immunobiology, The Immune System In Health and Disease, 2nd

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In preparing the nucleic acid sequence encoding the fusion polypeptide of the invention, the first, second, and third components of the fusion polypeptide are encoded in a single strand of nucleotides which, when expressed by a host vector system, produces a monomeric species of the fusion polypeptide. The monomers thus expressed then multimerize due to the interactions between the multimerizing components (the third fusion polypeptide components). Producing the fusion polypeptides in this manner avoids the need for purification of heterodimeric mixtures that would result if the first and second components were produced as separate molecules and then multimerized. For example, U.S. Patent No. 5,470,952 issued November 28, 1995 describes the production of heterodimeric proteins that function as CNTF or IL-6 antagonists. The heterodimers are purified from cell lines cotransfected with the appropriate alpha (α) and beta (β) components. Heterodimers are then separated from homodimers using methods such as passive elution from preparative, nondenaturing polyacrylamide gels or by using high pressure cation exchange chromatography. The need for this purification step is avoided by the methods of the present invention.

In addition, PCT International Application WO 96/11213 published 18 April 1996 entitled Dimeric IL-4 Inhibitors states that the applicant has prepared homodimers in which two IL-4 receptors are bound by a polymeric spacer and has prepared heterodimers in which an IL-4 receptor is linked by a polymeric spacer to an IL-2 receptor gamma chain. The polymeric spacer described is polyethylene glycol (PEG). The two receptor components, IL-4R and IL-2Rgamma are separately expressed and purified. Pegylated homodimers and heterodimers are then produced by joining the components together using bi-functional PEG reagents. It is an advantage

of the present invention that it avoids the need for such time consuming and costly purification and pegylation steps.

In one embodiment of the invention, the nucleotide sequence encoding the first component is upstream of the nucleotide sequence encoding the second component. In another embodiment of the invention, the nucleotide sequence encoding the first component is downstream of the nucleotide sequence encoding the second component. Further embodiments of the invention may be prepared in which the order of the first, second and third fusion polypeptide components are rearranged. For example, if the nucleotide sequence encoding the first component is designated 1, the nucleotide sequence encoding the second component is designated 2, and the nucleotide sequence of the third component is designated 3, then the order of the components in the isolated nucleic acid of the invention as read from 5′ to 3′ may be any of the following six combinations: 1,2,3; 1,3,2; 2,1,3; 2,3,1; 3,1,2; or 3,2,1.

In further embodiments of the invention, the cytokine bound by the fusion polypeptide may be a member of the hematopoietin family of cytokines selected from the group consisting of interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-9, interleukin-11, interleukin-13, interleukin-15, granulocyte macrophage colony stimulating factor, oncostatin M, leukemia inhibitory factor, and cardiotrophin-1.

In additional embodiments of the invention, the cytokine bound by the fusion polypeptide may be a member of the interferon family of cytokines selected from the group consisting of IFN-gamma, IFN-alpha, and IFN-beta.

In additional embodiments of the invention, the cytokine bound by the fusion polypeptide may be a member of the immunoglobulin superfamily

of cytokines selected from the group consisting of B7.1 (CD80) and B7.2 (B70).

In still further embodiments of the invention, the cytokine bound by the fusion polypeptide may be a member of the TNF family of cytokines selected from the group consisting of TNF-alpha, TNF-beta, LT-beta, CD40 ligand, Fas ligand, CD 27 ligand, CD 30 ligand, and 4-1BBL.

In additional embodiments of the invention, the cytokine bound by the fusion polypeptide may be a cytokine selected from the group consisting of interleukin-1, interleukin-10, interleukin-12, interleukin-14, interleukin-18, and MIF.

Because specificity determination and signal transduction occurs by a similar mechanism in the TGF-β/BMP family of cytokines (See D. Kingsley, Genes & Development, 1994, 8: 133-146; J. Wrana, Miner Electrolyte Metab, 24: 120-130 (1998); R. Derynck and X. Feng, Biochimica et Biophysica Acta 1333 (1997) F105-F150; and J. Massague and F. Weis-Garcia, "Serine/threonine Kinase Receptors: Mediators of Transforming Growth Factor Beta Family Signals" In Cancer Surveys, Vol. 27: Cell Signaling, 1996, Imperial Cancer Research Fund) the present invention may be used to produce high affinity antagonists for cytokines that are members of the TGF-β/BMP family.

Therefore, in additional embodiments of the invention, the cytokine bound by the fusion polypeptide may be a member of the TGF-β/BMP family selected from the group consisting of TGF-β1, TGF-β2, TGF-β3, BMP-2, BMP-3a, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8a, BMP-8b, BMP-9, BMP-10, BMP-11, BMP-15, BMP-16, endometrial bleeding associated factor (EBAF), growth differentiation factor-1 (GDF-1), GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-12, GDF-14, mullerian

inhibiting substance (MIS), activin-1, activin-2, activin-3, activin-4, and activin-5.

In alternative embodiments of the invention, the specificity determining component, the signal transducing component, or both, may be substituted for by a single chain Fv. A single chain Fv (scFv) is a truncated Fab having only the V region of a heavy chain linked by a stretch of synthetic peptide to a V region of a light chain. See, for example, US Patent Nos. 5,565,332; 5,733,743; 5,837,242; 5,858,657; and 5,871,907 assigned to Cambridge Antibody Technology Limited incorporated by reference herein. Thus the present invention contemplates, for example, an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex comprising a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the specificity determining component of the cytokine's receptor; a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence of an scFv capable of binding the cytokine at a site different from the site at which the cytokine binding portion of the extracellular domain of the specificity determining component of the cytokine's receptor binds; and a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component. Alternatively, the specificity determining component may be substituted for by a scFv that binds to a site on the cytokine different from the site at which the signal transducing component binds. Thus the invention contemplates an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex comprising a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of a scFv that binds to a site on the cytokine different from the site at which the cytokine binding portion of the extracellular domain of the signal transducing component of the cytokine's receptor binds; a nucleotide sequence encoding a second fusion polypeptide component

comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the signal transducing component of the cytokine's receptor; and a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component.

In another embodiment, the invention contemplates an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex comprising a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of a first scFv that binds to a site on the cytokine; a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence a second scFv that binds to a site on the cytokine different from the site at which the first scFv binds; and a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component.

In all of the above described embodiments comprising scFv's, the invention also contemplates embodiments in which the nucleotide sequence encoding the first component is upstream of the nucleotide sequence encoding the second component; embodiments in which the nucleotide sequence encoding the first component is downstream of the nucleotide sequence encoding the second component; and further embodiments of the invention in which the order of the first, second and third fusion polypeptide components is rearranged. For example, if the nucleotide sequence encoding the first component is designated 1, the nucleotide sequence encoding the second component is designated 2, and the nucleotide sequence of the third component is designated 3, then the order of the components in the isolated nucleic acid of the invention as read from 5' to 3' may be any of the following six combinations: 1,2,3; 1,3,2; 2,1,3; 2,3,1; 3,1,2; or 3,2,1.

In preferred embodiments of the invention, the multimerizing component comprises an immunoglobulin derived domain. More specifically, the immunoglobulin derived domain may be selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG. In another embodiment, the multimerizing component may be an Fc domain from which the first five amino acids (including a cysteine) have been removed to produce a multimerizing component referred to as Fc(ΔC1). Alternatively, the multimerizing component may be an Fc domain in which a cysteine within the first five amino acids has been substituted for by another amino acid such as, for example, serine or alanine.

The present invention also provides for fusion polypeptides encoded by the isolated nucleic acid molecules of the invention. Preferably, the fusion polypeptides are in multimeric form, due to the function of the third multimerizing component. In a preferred embodiment, the multimer is a dimer. Suitable multimerizing components are sequences encoding an immunoglobulin heavy chain hinge region (Takahashi et al., 1982, Cell 29:671-679); immunoglobulin gene sequences, and portions thereof. In a preferred embodiment of the invention, immunoglobulin gene sequences, especially one encoding the Fc domain, are used to encode the third multimerizing component.

The present invention also contemplates a vector which comprises the nucleic acid molecule of the invention as described herein.

Also provided is an expression vector comprising a nucleic acid molecule of the invention as described herein, wherein the nucleic acid molecule is operatively linked to an expression control sequence. Also provided is a host-vector system for the production of a fusion polypeptide which comprises the expression vector of the invention which has been introduced into a host cell suitable for expression of the fusion

polypeptide. The suitable host cell may be a bacterial cell such as E. coli, a yeast cell, such as Pichia pastoris, an insect cell, such as Spodoptera frugiperda, or a mammalian cell, such as a COS, CHO, 293, BHK or NS0 cell.

The present invention also provides for methods of producing the fusion polypeptides of the invention by growing cells of the host-vector systems described herein, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

The present invention provides novel antagonists which are based on receptor components that are shared by cytokines such as the CNTF family of cytokines.

The invention described herein contemplates the production of antagonists to any cytokine that utilizes an α specificity determining component which, when combined with the cytokine, binds to a first β signal transducing component to form a nonfunctional intermediate which then binds to a second β signal transducing component causing β-receptor dimerization and consequent signal transduction. According to the invention, the soluble α specificity determining component of the receptor (sRα) and the extracellular domain of the first β signal transducing component of the cytokine receptor (β1) are combined to form heterodimers (sRα:β1) that act as antagonists to the cytokine by binding the cytokine to form a nonfunctional complex.

As described in Example 1, CNTF and IL-6 share the β1 receptor component gp130. The fact that CNTF forms an intermediate with CNTFRα and gp130 can be demonstrated (Example 1) in cells lacking LIFRβ, where the complex of CNTF and CNTFRα binds gp130, and

prevents homodimerization of gp130 by IL-6 and IL-6Rα, thereby blocking signal transduction. These studies provide the basis for the development of the IL-6 antagonists described herein, as they show that if, in the presence of a ligand, a nonfunctional intermediate complex, consisting of the ligand, its α receptor component and its β1 receptor component, can be formed, it will effectively block the action of the ligand. Other cytokines may use other β1 receptor components, such as LIFRβ, which may also be used to produce antagonists according to the present invention.

Thus for example, in one embodiment of the invention, effective antagonists of IL-6 or CNTF consist of heterodimers of the extracellular domains of the α specificity determining components of their receptors (sIL-6Rα and sCNTFRα, respectively) and the extracellular domain of gp130. The resultant heterodimers, which are referred to hereinafter as sIL-6Rα:β1 and sCNTFRα:β1, respectively, function as high-affinity traps for IL-6 or CNTF, respectively, thus rendering the cytokine inaccessible to form a signal transducing complex with the native membrane-bound forms of their receptors.

Although soluble ligand binding domains from the extracellular portion of receptors have proven to be somewhat effective as traps for their ligands and thus act as antagonists [Bargetzi, et al., Cancer Res. 53:4010-4013 (1993); , et al., Proc. Natl. Acad. Sci. USA 89: 8616-8620 (1992); Mohler, et al., J. Immunol. 151: 1548-1561 (1993); Narazaki, et al., Blood 82: 1120-1126 (1993)], the IL-6 and CNTF receptors are unusual in that the α receptor components constitute ligand binding domains that, in concert with their ligands, function effectively in soluble form as receptor agonists [Davis, et al. Science 259:1736-1739 (1993); Taga, et al., Cell 58: 573-581 (1989)]. The sRα:β1 heterodimers prepared according to the present invention provide effective traps for their ligands, binding these ligands with affinities in the picomolar range (based on binding studies for CNTF to PC12D cells)

without creating functional intermediates. The technology described herein may be applied to develop a cytokine trap for any cytokine that utilizes an α-component that confers specificity, as well as a β component which, when bound to the α-specificity component, has a higher affinity for the cytokine than either component alone. Accordingly, antagonists according to the invention include antagonists of interleukins 1 through 5 [IL-1, Greenfeder, et al. J. Biol. Chem. 270:13757-13765 (1995); Guo, et al. J. Biol. Chem. 270:27562-27568 (1995)], IL-2; [Taniguchi, et al. European Patent Nos. 0386289-A and 0386304-A (1990); Takeshita, et al. Science 257:379-382 (1992)]; IL-3; [Kitamura, et al. Cell 66:1165-1174 (1991)], IL-4; [Idzerda, et al. J. Exp. Med. 171:861-873 (1990)], IL-5; [Taverneir, et al. Cell 66:1175-1184 (1991)], IL-11 [(Cherel, et al. Direct Submission to EMBL/GenBank/DDBJ databases; accession No. Z38102)], interleukin 15 [IL-15; Hemar, et al. J. Cell Biol. 1295:55-64 (1995); Taniguchi, et al. European Patent Nos. 0386289-A and 0386304-A (1990); Takeshita, et al. Science 257:379-382 (1992)], granulocyte-macrophage colony stimulating factor [GM-CSF; Hayashida, et al. Proc. Natl. Acad. Sci. U.S.A. 97:9655-9659 (1990)], LIF, gamma interferon [IFNγ; Aguet, et al. Cell 55:273-280 (1988); Soh, et al. Cell 76:793-802 (1994)], and transforming growth factor beta [TGFβ; Inagaki, et al. Proc. Natl. Acad. Sci. USA 90:5359-5363 (1993)].

The α and β receptor extracellular domains may be prepared using methods known to those skilled in the art. The CNTFRα receptor has been cloned, sequenced and expressed [Davis, et al. (1991) Science 253:59-63 which is incorporated by reference in its entirety herein]. The cloning of LIFRβ and gp130 are described in Gearing et al. in EMBO J. 10:2839-2848 (1991), Hibi, et al. Cell 63:1149-1157 (1990) and in published PCT application WO 93/10151 published May 27, 1993, all of which are incorporated by reference in their entirety herein.

The receptor molecules useful for practicing the present invention may be prepared by cloning and expression in a prokaryotic or eukaryotic expression system. The recombinant receptor gene may be expressed and purified utilizing any number of methods. The gene encoding the factor may be subcloned into a bacterial expression vector, such as for example, but not by way of limitation, pCP110.

The recombinant factors may be purified by any technique which allows for the subsequent formation of a stable, biologically active protein. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

The sRα:β heterodimeric receptors may be engineered using known fusion regions, as described in published PCT application WO 93/10151 published May 27, 1993 entitled "Receptor for Oncostatin M and Leukemia Inhibitory Factor" which describes production of β receptor heterodimers, or they may be prepared by crosslinking of extracellular domains by chemical means. The domains utilized may consist of the entire extracellular domain of the α and β components, or they may consist of mutants or fragments thereof that maintain the ability to form a complex with its ligand and other components in the sRα:β1 complex. For example, as described below in Example 4, IL-6 antagonists have been prepared using gp130 that is lacking its three fibronectin-like domains.

In one embodiment of the invention, the extracellular domains are engineered using leucine zippers. The leucine zipper domains of the human transcription factors c-jun and c-fos have been shown to form stable heterodimers [Busch and Sassone-Corsi, Trends Genetics 6: 36-40

(1990); Gentz, et al., Science 243: 1695-1699 (1989)] with a 1:1 stoichiometry. Although jun-jun homodimers have also been shown to form, they are about 1000-fold less stable than jun-fos heterodimers. Fos-fos homodimers have not been detected.

The leucine zipper domain of either c-jun or c-fos are fused in frame at the C-terminus of the soluble or extracellular domains of the above mentioned receptor components by genetically engineering chimeric genes. The fusions may be direct or they may employ a flexible linker domain, such as the hinge region of human IgG, or polypeptide linkers consisting of small amino acids such as glycine, serine, threonine or alanine, at various lengths and combinations. Additionally, the chimeric proteins may be tagged by His-His-His-His-His-His (His6),[SEQ. ID NO. 1] to allow rapid purification by metal-chelate chromatography, and/or by epitopes to which antibodies are available, to allow for detection on western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

In another embodiment, as described below in Example 3, the sRα:β1 heterodimer is prepared using a similar method, but using the Fc-domain of human IgG1 [Aruffo, et al., Cell 67:35-44 (1991)]. In contrast to the latter, formation of heterodimers must be biochemically achieved, as chimeric molecules carrying the Fc-domain will be expressed as disulfide-linked homodimers. Thus, homodimers may be reduced under conditions that favor the disruption of inter-chain disulfides but do not effect intra-chain disulfides. Then monomers with different extracellular portions are mixed in equimolar amounts and oxidized to form a mixture of homo- and heterodimers. The components of this mixture are separated by chromatographic techniques. Alternatively, the formation of this type of heterodimers may be biased by genetically engineering and expressing molecules that consist of the soluble or extracellular portion of the receptor components followed by the Fc-domain of hIgG, followed by

either the c-jun or the c-fos leucine zippers described above [Kostelny, et al., J. Immunol. 148: 1547-1553 (1992)]. Since these leucine zippers form predominately heterodimers, they may be used to drive formation of the heterodimers where desired. As for the chimeric proteins described using leucine zippers, these may also be tagged with metal chelates or an epitope. This tagged domain can be used for rapid purification by metal-chelate chromatography, and/or by antibodies, to allow for detection on western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

In additional embodiments, heterodimers may be prepared using other immunoglobulin derived domains that drive the formation of dimers. Such domains include, for example, the heavy chains of IgG (Cγ1 and Cγ4), as well as the constant regions of kappa (κ) and lambda (λ) light chains of human immunoglobulins. The heterodimerization of Cγ with the light chain occurs between the CH1 domain of Cγ and the constant region of the light chain (CL), and is stabilized by covalent linking of the two domains via a single disulfide bridge. Accordingly, as described in Example 4, constructs may be prepared using these immunoglobulin domains. Alternatively, the immunoglobulin domains include domains that may be derived from T cell receptor components which drive dimerization. In another embodiment of the invention, the sRα:β1 heterodimers are prepared by expression as chimeric molecules utilizing flexible linker loops. A DNA construct encoding the chimeric protein is designed such that it expresses two soluble or extracellular domains fused together in tandem ("head to head") by a flexible loop. This loop may be entirely artificial (e.g. polyglycine repeats interrupted by serine or threonine at a certain interval) or "borrowed" from naturally occurring proteins (e.g. the hinge region of hIgG). Molecules may be engineered in which the order of the soluble or extracellular domains fused is switched (e.g. sIL6Rα/loop/sgp130 or sgp130/loop/sIL-6Rα) and/or in which the length

and composition of the loop is varied, to allow for selection of molecules with desired characteristics.

Alternatively, the heterodimers made according to the present invention may be purified from cell lines cotransfected with the appropriate α and β components. Heterodimers may be separated from homodimers using methods available to those skilled in the art. For example, limited quantities of heterodimers may be recovered by passive elution from preparative, nondenaturing polyacrylamide gels. Alternatively, heterodimers may be purified using high pressure cation exchange chromatography. Excellent purification has been obtained using a Mono S cation exchange column.

In addition to sRα:β1 heterodimers that act as antagonists by binding free CNTF or IL-6, the present invention also contemplates the use of engineered, mutated versions of IL-6 with novel properties that allow it to bind to IL-6Rα and a single gp130 molecule, but fail to engage the second gp130 to complete β component homodimerization, and thus act as an effective IL-6 antagonist on any IL-6 responsive cell. Our model for the structure of the IL-6 and CNTF receptor complexes indicates that these cytokines have distinct sites for binding the α, β1, and β2 receptor components [Stahl and Yancopoulos, Cell 74: 587-590 (1993)]. Mutations of critical amino acid residues comprising each of these sites gives rise to novel molecules which have the desired antagonistic properties. Ablation of the β1 site would give a molecule which could still bind to the α receptor component but not the β1 component, and thereby comprise an antagonist with nanomolar affinity. Mutations of critical amino acid residues comprising the β2 site of IL-6 (IL-6β2$^-$) would give a molecule that would bind to IL-6Rα and the first gp130 monomer, but fail to engage the second gp130 and thus be functionally inactive. Similarly, mutations of

the CNTF β2 site would give a molecule (CNTFβ2⁻) that would bind CNTFRα and gp130, but fail to engage LIFRβ, thereby antagonizing CNTF action by forming the non-functional β1 intermediate. Based on the binding results described above where CNTF forms the β1 intermediate with high affinity, both CNTFβ2⁻ and IL-6β2⁻ would constitute antagonists with affinity in the range of 10 pM.

A variety of means are used to generate and identify mutations of IL-6 or CNTF that have the desired properties. Random mutagenesis by standard methods of the DNA encoding IL-6 or CNTF may be used, followed by analysis of the collection of products to identify mutated cytokines having the desired novel properties as outlined below. Mutagenesis by genetic engineering has been used extensively in order to elucidate the structural organization of functional domains of recombinant proteins. Several different approaches have been described in the literature for carrying out deletion or substitution mutagenesis. The most successful appear to be alanine scanning mutagenesis [Cunningham and Wells (1989), Science 244: 1081-1085] and homolog-scanning mutagenesis [Cunningham, et al., (1989), Science 243:1330-1336].

Targeted mutagenesis of the IL-6 or CNTF nucleic acid sequences using such methods can be used to generate CNTFβ2- or IL-6β2- candidates. The choice of regions appropriate for targeted mutagenesis is done systematically, or determined from studies whereby panels of monoclonal antibodies against each factor are used to map regions of the cytokine that might be exposed after binding of the cytokine to the α receptor component alone, or to the αβ1 heterodimeric soluble receptors described above. Similarly, chemical modification or limited proteolysis of the cytokine alone or in a complex bound to the α receptor component or the αβ1 heterodimeric soluble receptors described above, followed by analysis

of the protected and exposed regions could reveal potential β2 binding sites.

Assays for identifying CNTF or IL-6 mutants with the desired properties involve the ability to block with high affinity the action of IL-6 or CNTF on appropriately responsive cell lines [Davis, et al., Science 259: 1736-1739 (1993); Murakami, et al., Proc. Natl. Acad. Sci. USA 88: 11349-11353 (1991)]. Such assays include cell proliferation, survival, or DNA synthesis driven by CNTF or IL-6, or the construction of cell lines where binding of factor induces production of reporters such as CAT or β–galactosidase [Savino, et al., Proc. Natl. Acad. Sci. USA 90: 4067-4071 (1993)].

Alternatively, the properties of various mutants may be assessed with a receptor-based assay. One such assay consists of screening mutants for their ability to bind the sRα:β1 receptor heterodimers described above using epitope-tagged [Davis et al., Science 253: 59-63 (1991)] sRα:β1 reagents. Furthermore, one can probe for the presence or absence of the β2 site by assessing whether an epitope-tagged soluble β2 reagent will bind to the cytokine in the presence of the β1 heterodimer. For example, CNTF only binds to LIFRβ (the β2 component) in the presence of both CNTFRα and gp130 [Davis, et al. Science 260: 1805-1808 (1993); Stahl, et al. J. Biol. Chem. 268: 7628-7631 (1993)]. Thus a soluble LIFRβ reagent would only bind to CNTF in the presence of the soluble sRα:β1 dimer sCNTFRα:β1. For IL-6, the sRα:β1 reagent would be IL-6Rα:β1, and the probe for the β2 site would be epitope-tagged sgp130. Thus $\beta2^-$ mutants of CNTF would be identified as those that bound the sRα:β1 reagent, demonstrating that the α and β1 site of the cytokine were intact, yet failed to bind the β2 reagent.

In addition, the present invention provides for methods of detecting or measuring the activity of potential β2- mutants by measuring the phosphorylation of a β-receptor component or a signal transduction component selected from the group consisting of Jak1, Jak2 and Tyk2 or any other signal transduction component, such as the CLIPs, that are determined to be phosphorylated in response to a member of the CNTF family of cytokines.

A cell that expresses the signal transduction component(s) described herein may either do so naturally or be genetically engineered to do so. For example, Jak1 and Tyk-2-encoding nucleic acid sequences obtained as described in Velazquez, et al., Cell, Vol. 70:313-322 (1992), may be introduced into a cell by transduction, transfection, microinjection, electroporation, via a transgenic animal, etc., using any known method known in the art.

According to the invention, cells are exposed to a potential antagonist and the tyrosine phosphorylation of either the β-component(s) or the signal transduction component(s) are compared to the tyrosine phosphorylation of the same component(s) in the absence of the potential antagonist. In another embodiment of the invention, the tyrosine phosphorylation that results from contacting the above cells with the potential antagonist is compared to the tyrosine phosphorylation of the same cells exposed to the parental CNTF family member. In such assays, the cell must either express the extracellular receptor (α-component) or the cells may be exposed to the test agent in the presence of the soluble receptor component. Thus, for example, in an assay system designed to identify agonists or antagonists of CNTF, the cell may express the α- component CNTFRα, the β-components gp130 and LIFRβ and a signal transducing component such as Jak1. The cell is exposed to test agents, and the tyrosine phosphorylation of either the β- components or the signal transducing component is

compared to the phosphorylation pattern produced in the presence of CNTF. Alternatively, the tyrosine phosphorylation which results from exposure to a test agent is compared to the phosphorylation which occurs in the absence of the test agent. Alternatively, an assay system, for example, for IL-6 may involve exposing a cell that expresses the β-component gp130 and a signal transducing protein such as Jak1, Jak2 or Tyk2 to a test agent in conjunction with the soluble IL-6 receptor.

In another embodiment of the invention the above approaches are used to develop a method for screening for small molecule antagonists that act at various steps in the process of ligand binding, receptor complex formation, and subsequent signal transduction. Molecules that potentially interfere with ligand-receptor interactions are screened by assessing interference of complex formation between the soluble receptors and ligand as described above. Alternatively, cell-based assays in which IL-6 or CNTF induce response of a reporter gene are screened against libraries of small molecules or natural products to identify potential antagonists. Those molecules showing antagonist activity are rescreened on cell-based assays responding to other factors (such as GM-CSF or factors like Neurotrophin-3 that activate receptor tyrosine kinases) to evaluate their specificity against the CNTF/IL-6/OSM/LIF family of factors. Such cell-based screens are used to identify antagonists that inhibit any of numerous targets in the signal transduction process.

In one such assay system, the specific target for antagonists is the interaction of the Jak/Tyk family of kinases [Firmbach-Kraft, Oncogene 5: 1329-1336 (1990); Wilks, et al., Mol. Cell. Biol. 11:2057-2065 (1991)] with the receptor β subunits. As described above, LIFRβ and gp130 preassociate with members of the Jak/Tyk family of cytoplasmic protein tyrosine kinases, which become activated in response to ligand-induced β component dimerization Stahl, et al. Science 263:92-95 (1993). Thus small molecules that could enter the cell cytoplasm and disrupt the interaction

between the β component and the Jak/Tyk kinase could potentially block all subsequent intracellular signaling. Such activity could be screened with an *in vitro* scheme that assessed the ability of small molecules to block the interaction between the relevant binding domains of purified β component and Jak/Tyk kinase. Alternatively, one could easily screen for molecules that could inhibit a yeast-based assay of β component binding to Jak/Tyk kinases using the two-hybrid interaction system [Chien, et al., Proc. Natl. Acad. Sci. 88: 9578-9582 (1991)]. In such a system, the interaction between two proteins (β component and Jak/Tyk kinase or relevant domains thereof in this example) induces production of a convenient marker such as β- galactosidase. Collections of small molecules are tested for their ability to disrupt the desired interaction without inhibiting the interaction between two control proteins. The advantage of this screen would be the requirement that the test compounds enter the cell before inhibiting the interaction between the β component and the Jak/Tyk kinase.

The CNTF family antagonists described herein either bind to, or compete with the cytokines CNTF and IL-6. Accordingly, they are useful for treating diseases or disorders mediated by CNTF or IL-6. For example, therapeutic uses of IL-6 antagonists would include the following:

1) In osteoporosis, which can be exacerbated by lowering of estrogen levels in post-menopausal women or through ovariectomy, IL-6 appears to be a critical mediator of osteoclastogenesis, leading to bone resorption [Horowitz, Science 260: 626-627 (1993); Jilka, et al., Science 257: 88-91 (1992)]. Importantly, IL-6 only appears to play a major role in the estrogen-depleted state, and apparently is minimally involved in normal bone maintenance. Consistent with this, experimental evidence indicates that function-blocking antibodies to IL-6 can reduce the number of osteoclasts [Jilka, et al. Science 257: 88-91 (1992)]. While estrogen replacement therapy is also used, there appear to be side effects that may include increased risk of

endometrial and breast cancer. Thus, IL-6 antagonists as described herein would be more specific to reduce osteoclastogenesis to normal levels.

2) IL-6 appears to be directly involved in multiple myeloma by acting in either an autocrine or paracrine fashion to promote tumor formation [van Oers, et al., Ann Hematol. 66: 219-223 (1993)]. Furthermore, the elevated IL-6 levels create undesirable secondary effects such as bone resorption, hypercalcemia, and cachexia; in limited studies function-blocking antibodies to IL-6 or IL-6Ra have some efficacy [Klein, et al., Blood 78: 1198-1204 (1991); Suzuki, et al., Eur. J. Immunol. 22:1989-1993 (1992)]. Therefore, IL-6 antagonists as described herein would be beneficial for both the secondary effects as well as for inhibiting tumor growth.

3) IL-6 may be a mediator of tumor necrosis factor (TNF) that leads to cachexia associated with AIDS and cancer [Strassmann, et al., J. Clin. Invest. 89: 1681-1684 (1992)], perhaps by reducing lipoprotein lipase activity in adipose tissue [Greenberg, et al., Cancer Research 52: 4113-4116 (1992)]. Accordingly, antagonists described herein would be useful in alleviating or reducing cachexia in such patients.

Effective doses useful for treating these or other CNTF family related diseases or disorders may be determined using methods known to one skilled in the art [see, for example, Fingl, et al., The Pharmacological Basis of Therapeutics, Goodman and Gilman, eds. Macmillan Publishing Co., New York, pp. 1-46 ((1975)]. Pharmaceutical compositions for use according to the invention include the antagonists described above in a pharmacologically acceptable liquid, solid or semi-solid carrier, linked to a carrier or targeting molecule (e.g., antibody, hormone, growth factor, etc.) and/or incorporated into liposomes, microcapsules, and controlled release preparation (including antagonist expressing cells) prior to administration *in vivo*. For example, the pharmaceutical composition may comprise one or more of the antagonists in an aqueous solution, such as sterile water, saline, phosphate buffer or dextrose solution. Alternatively, the active agents may be comprised in a solid (e.g. wax) or semi-solid (e.g. gelatinous) formulation that may be implanted into a patient in need of such

treatment. The administration route may be any mode of administration known in the art, including but not limited to intravenously, intrathecally, subcutaneously, by injection into involved tissue, intraarterially, intranasally, orally, or via an implanted device.

Administration may result in the distribution of the active agent of the invention throughout the body or in a localized area. For example, in some conditions which involve distant regions of the nervous system, intravenous or intrathecal administration of agent may be desirable. In some situations, an implant containing active agent may be placed in or near the lesioned area. Suitable implants include, but are not limited to, gelfoam, wax, or microparticle-based implants.

EXAMPLES

EXAMPLE 1: CNTF COMPETES WITH IL-6 FOR BINDING TO GP130

MATERIALS AND METHODS

Materials. A clone of PC12 cells that respond to IL-6 (PC12D) was obtained from DNAX. Rat CNTF was prepared as described [Masiakowski, et al., J. Neurochem. 57:1003-10012 (1991)]. IL-6 and sIL-6Rα were purchased from R & D Systems. Antisera was raised in rabbits against a peptide derived from a region near the C-terminus of gp130 (sequence: CGTEGQVERFETVGME) [SEQ. ID. NO. 2] by the method described (Stahl, et al. J. Biol. Chem. 268:7628-7631 (1993). Anti-phosphotyrosine monoclonal 4G10 was purchased from UBI, and reagents for ECL from Amersham.

Signal Transduction Assays. Plates (10 cm) of PC12D were starved in serum-free medium (RPMI 1640 + glutamine) for 1 hour, then incubated with IL-6 (50 ng/mL) + sIL-6R (1 mg/mL) in the presence or absence of

added rat CNTF at the indicated concentrations for 5 minutes at 37°C. Samples were then subjected to anti-gp130 immunoprecipitation, SDS PAGE, and anti-phosphotyrosine immunoblotting as described (Stahl, et al. J. Biol. Chem. 268:7628-7631 (1993).

RESULTS

The ability of CNTF to block IL-6 responses was measured using a PC12 cell line (called PC12D) that expresses IL-6Rα, gp130, and CNTFRα, but not LIFRβ. As one would predict, these cells respond to IL-6, but not to CNTF (Fig. 2) since LIFRβ is a required component for CNTF signal transduction [Davis, et al., Science 260: 59-63 (1993)]. In accordance with results on other cell lines [Ip, et al., Cell 69: 1121-1132 (1992)], PC12D cells give tyrosine phosphorylation of gp130 (as well as a variety of other proteins called CLIPs) in response to 2 nM IL-6 (Fig. 2). Addition of recombinant soluble IL-6Rα (sIL-6Rα) enhances the level of gp130 tyrosine phosphorylation, as has been reported in some other systems [(Taga, et al., Cell 58: 573-581 (1989)]. However, addition of 2 nM CNTF simultaneously with IL-6 severely diminishes the tyrosine phosphorylation of gp130. Although a slight gp130 phosphorylation response remains in the presence of CNTF, IL-6, and sIL-6Rα, it is eliminated if the CNTF concentration is increased fourfold to 8 nM. Thus, in IL-6 responsive cells that contain CNTFRα but no LIFRβ, CNTF is a rather potent antagonist of IL-6 action.

EXAMPLE 2. BINDING OF CNTF TO THE CNTFRα:β

MATERIALS AND METHODS

Scatchard Analysis of CNTF Binding. ^{125}I-CNTF was prepared and purified as described [Stahl et al. JBC 268: 7628-7631 (1993)]. Saturation binding studies were carried out in PC12 cells, using concentrations of ^{125}I-

CNTF ranging from 20pM to 10nM. Binding was performed directly on a monolayer of cells. Medium was removed from wells and cells were washed once with assay buffer consisting of phosphate buffered saline (PBS; pH 7.4), 0.1mM bacitracin, 1mM PMSF, 1mg/ml leupeptin, and 1mg/ml BSA. Cells were incubated in ^{125}I-CNTF for 2 hours at room temperature, followed by 2 quick washes with assay buffer. Cells were lysed with PBS containing 1% SDS and counted in a Packard Gamma Counter at 90-95% efficiency. Non-specific binding was defined by the presence of 100-fold excess of unlabelled CNTF. Specific binding ranged from 70% to 95%.

<u>RESULTS</u>

The equilibrium constant for binding of CNTF to CNTFRα:β1 was estimated from Scatchard analysis of iodinated CNTF binding on PC12D cells (Figure 3). The data is consistent with a 2 site fit having dissociation constants of 9 pM and 3.4 nM. The low affinity site corresponds to interaction of CNTF with CNTFRα , which has a Kd near 3 nM [(Panayotatos, et al., J. Biol. Chem. 268: 19000-19003 (1993)]. We interpret the high affinity complex as the intermediate containing CNTF, CNTFRα , and gp130. A Ewing sarcoma cell line (EW-1) which does contain CNTFRα, gp130, and LIFRβ, and therefore gives robust tyrosine phosphorylation in response to CNTF, displays a very similar two site fit with dissociation constants of 1 nM and 10. Thus it is apparent that CNTF binds with equally high affinity to a complex containing only CNTFRα and gp130, as it does to a complex which additionally contains LIFRβ, thus demonstrating the feasibility of creating the sRα:β antagonists described herein.

EXAMPLE 3. METHODS OF PRODUCING CYTOKINE LIGAND TRAPS

Virus Stock Production

SF21 insect cells obtained from *Spodoptera frugiperda* were grown at 27°C in Gibco SF900 II medium to a density of 1×10^6 cells/mL. The individual virus stock for either GP130-Fc-His$_6$ (Figure 4) or IL6Ra-Fc (Figure 5) was added to the bioreactor to a low multiplicity 0.01-0.1 PFU/cell to begin the infection. The infection process was allowed to continue for 5-7 days allowing maximum virus replication without incurring substantial cell lysis. The cell suspension was aseptically aliquoted into sterile centrifuge bottles and the cells removed by centrifugation. The cell-free supernatant was collected in sterile bottles and stored at 4°C until further use.

The virus titer was determined by plaque assay as described by O'Reilly, Miller and Luckow. The method is carried out in 60mm tissue-culture dishes which are seeded with 2×10^6 cells. Serial dilutions of the virus stock are added to the attached cells and the mixture incubated with rocking to allow the virus to adsorb to individual cells. An agar overlay is added and plates incubated for 5 - 7 days at 27°C. Staining of viable cells with neutral red revealed circular plaques resulting which were counted to give the virus titer.

Coinfection of Cells for Protein Production

Uninfected SF21 Cells were grown in a 60L ABEC bioreactor containing 40L of SF900 II medium. Temperature was controlled at 27°C and the dissolved oxygen level was maintained at 50% of saturation by controlling the flowrate of oxygen in the inlet gas stream. When a density of 2×10^6 cells/mL was reached, the cells were concentrated within the bioreactor to a volume of 20L using a low shear steam sterilizable pump with a tangential flow filtration device with Millipore Prostak 0.65 micron

membranes. After concentration fresh sterile growth medium is slowly added to the bioreactor while the filtration system continues to remove the spent growth medium by diafiltration. After two volume exchanges (40L) have been carried out an additional 20L of fresh medium was added to the bioreactor to resuspend the cells to the original volume of 40L. The cell density was determined once again by counting viable cells using a hemacytometer.

The required amount of each virus stock was calculated based on the cell density, virus titer and the desired multiplicity of infection (MOI). Virus stock ratios of 5:1, 5:2, 10:2 and 10:4, IL6Rα-Fc to GP130-Fc-His_6 all resulted in production of significant amounts of heterodimer. The ideal virus stock ratio is highly dependent on the ease of purification of the heterodimer from each of the two homodimers. The IL6Rα-Fc homodimer is relatively easy to remove downstream by immobilized metal affinity chromatography. Virus infection ratios have been chosen to minimize the formation of the GP130-Fc-His_6 homodimer which is more difficult to clear downstream. The relative amount of GP130-Fc-His_6 virus stock chosen for infection has increased with successive batches as the purification method for clearing the resultant homodimer has improved.

The virus stocks were aseptically mixed in a single vessel then transferred to the bioreactor. This results in synchronous infection of the SF21 cells. The infection is allowed to proceed for three to four days, allowing sufficient time for maximal production of the heterodimer protein.

Recovery and Protein A Chromatographic Purification

At the conclusion of the infection phase of the bioreactor process the cells were concentrated in the bioreactor using a 10 ft^2 Millipore Prostak filter (0.65 micron) pore size. The cell-free permeate passing through the filter was collected in a clean process vessel. At the conclusion of the filtration

operation the pH of permeate stream, containing the protein product, was adjusted to 8.0 with 10N NaOH. The resultant precipitate was removed by forcing the extract through a 0.8 micron depth filter (Sartorious), followed by a 0.2 micron filter. Sufficient 0.5M EDTA stock was added to give a final concentration of 5mM. The filtered protein solution was loaded onto a 10 cm diameter column containing 100-200 mL of Pharmacia Protein A Sepharose 4 Fast Flow, equilibrated with PBS. Protein A has a very high affinity for the Fc-Fc domain of each of the 3 recombinant protein products, allowing them to bind while other proteins in the cell-free extract flow through the column. After loading the column was washed to baseline with PBS containing an additional 350mM NaCl. The IgG-Fc tagged proteins were eluted at low pH, either with 0.5M acetic acid or with a decreasing pH gradient of 0.1M citric acid and 0.2M disodium phosphate buffers. Tris base or disodium phosphate was added to the eluted protein to avoid prolonged exposure to low pH conditions.

The pooled protein was diafiltered into PBS or HEPES buffer and derivitized with 1 mM iodoacetamide to protect the exposed sulfhydryl group on the free cysteine near the hinge region of each Fc domain. This prevents disulfide mediated aggregation of proteins. A 6 ft^2 Millipore spiral wound ultrafiltration membrane with nominal 30 kiloDalton cutoff was used to perform the buffer exchange. The total protein was determined by UV absorbance at 280 nm using the diafiltration buffer as a blank. The relative amounts of heterodimer and two homodimer proteins were determined by SDS PAGE gel electrophoresis using a 6% Tris-Glycine gel (Novex). Gels were Coomassie-stained then transferred into destain solution overnight. A Shimadzu scanning densitometer was used to determine the relative intensity of the individual protein bands on the SDS PAGE gel. The peak area ratios are used to compute the fraction of heterodimer and each of the homodimers in the column pool fractions.

Immobilized Metal Affinity Chromatographic Purification

The six histidine residues on the C-terminus of the GP130-Fc-His_6 fusion protein provides an excellent molecular handle for separation of the heterodimeric IL6 antagonist from the two homodimers. The imidazole group on each of the C-terminal histidines of the GP130-Fc-His_6 moiety has a strong binding constant with several divalent metals, including copper, nickel, zinc, cobalt, iron and calcium. Since the IL6Rα-Fc homodimer has no C-terminal histidine residues, it clearly has the lowest affinity. The IL6Rα-Fc-GP130-Fc-His_6 heterodimer has a single stand set six histidines giving it greater affinity for the metal, while the GP130-Fc-His_6 homodimer has two sets of six histidines each giving it the highest affinity of the three IgG tagged proteins to the metal affinity column. Selective elution of the three proteins with increasing amounts of imidazole in the elution buffer therefore elutes the proteins in the following order:

1. IL6Rα-Fc homodimer
2. IL6Rα-Fc-GP130-Fc-His heterodimer
3. GP130-Fc-His homodimer

A 26 mm diameter column containing 100 mL of Pharmacia Chelating Sepharose Fast Flow was saturated with a solution of nickel sulfate until a significant green color is observed in the column eluate. The column is then washed with several column volumes of deionized water, then equilibrated with 50 mM HEPES, 40mM imidazole, pH 8.0. The binding of imidazole to the immobilized nickel results in a green to blue color change. Imidazole was added to the protein load to a final concentration of 40mM. Addition of imidazole to the protein load reduces the binding of IL6Rα-Fc homodimer, increasing the surface area available for the remaining two species. After loading, the column was washed with

several column volumes of 50 mM HEPES, 80mM imidazole, pH 8.0 until a steady baseline was reestablished. The heterodimer was selectively eluted with 50 mM HEPES, 150mM imidazole, pH 8.0 over several column volumes. The protein fractions were pooled and diafiltered into PBS as described in the section above.

EXAMPLE 4. ALTERNATIVE METHODS OF CONSTRUCTING LIGAND TRAPS

As described above, receptor activation by CNTF, and analogously by IL-6 and IL-11, follows an ordered sequence of binding events (Figure 6). The cytokine initially binds to its cognate Rα with low affinity (Kd = 3 to 10 nM); this is a required step - cells which do not express the cognate Rα do not respond to the cognate cytokine. The cytokine•Rα complex associates with the first signal transducing component, gp130, to form a high affinity complex (Kd in the order of 10 pM for the CNTF•CNTFRα•gp130 complex). This complex does not transduce signal, as it is the dimerization of the signal transducing components that brings about signaling (Stahl and Yancopoulos, J. Neurobiology 25: 1454-1466 (1994); Stahl et al., Science 267:1349-1353 (1995); Davis et al., Science 260:1805-1808 (1993); Stahl et al., Science 263:92-95 (1994); Murakami, et al. Science 260:1808-1810 (1993). At least in the case of IL-6, the cytokine•Rα•signal transducer heterotrimeric complex subsequently associates with another like complex, to form a hexameric complex (Figure 6) (Ward et al., J. Biol. Chem. 269:23286-23289 (1994). The resulting dimerization of the signal transducers - gp130 in the case of IL-6 (Murakami et al., Science 260:1808-1810 (1993) and IL-11, gp130 and LIFR in the case of CNTF (Davis et al., Science 260:1805-1808 (1993) - brings about signal transduction.

The initial heterodimeric molecules made comprised a soluble Rα-component linked to the extracellular domain of gp130. These molecules

were shown to mimic the high affinity cytokine•Rα•gp130 complex and behave as a high affinity antagonist of their cognate cytokine (Figure 7). To make these molecules, the extracellular domain of gp130 was paired with the extracellular domain of the α-receptor components for IL-6 and CNTF, IL-6Rα and CNTFRα respectively. To link the Rα with the extracellular domain of gp130, the soluble Rα-components and gp130 were fused to the Fc portion of human IgG1 to produce Rα-Fc and gp130-Fc respectively. The Fc domain was chosen primarily but not solely because it naturally forms disulfide-linked dimers. Heterodimeric molecules comprising Rα–Fc•gp130-Fc were expressed, purified and shown to behave as highly potent antagonists of their cognate ligand. Furthermore, these molecules were found to be highly specific for their cognate cytokine since it is the choice of the α–receptor component which specifies which cytokine is bound and trapped (there is no measurable binding of the cytokine to gp130 in the absence of the appropriate Rα).

Here we describe an extension of this technology which allows the engineering of different heteromeric soluble receptor ligand traps which by virtue of their design may have additional beneficial characteristics such as stability, Fc-receptor-mediated clearance, or reduced effector functions (such as complement fixation). Furthermore, the technology described should prove suitable for the engineering of any heteromeric protein in mammalian or other suitable protein expression systems, including but not limited to heteromeric molecules which employ receptors, ligands, and catalytic components such as enzymes or catalytic antibodies.

MATERIALS AND METHODS

Genetic engineering of heteromeric immunoglobulin heavy/light chain soluble receptor-based ligand traps for IL-6.

The IL-6 traps described here were engineered using human gp130, human IL-6 α-receptor (IL-6Rα), the constant region of the heavy chains (**Cγ**) of human IgG1 (**Cγ1**) (Lewis et al., Journal of Immunology 151:2829-2838 (1993) or IgG4 (**Cγ4**) with or without a join-region (**J**), and the constant regions of kappa (**κ**) and lambda (**λ**) (Cheung, et al., Journal of Virology 66:6714-6720 (1992) light chains of human immunoglobulin (**Ig**), also with or without a different j-peptide (**j**). This design takes advantage of the natural ability of the Cγ domain to heterodimerize with κ or λ light chains. The heterodimerization of Cγ with the light chain occurs between the CH1 domain of Cγ and the constant region of the light chain (C_L), and is stabilized by covalent linking of the two domains via a single disulfide bridge. We reasoned that, like the Fc domain of human IgG1, the combination of Cγ with C_L could be used to produce disulfide linked heteromeric proteins comprised of the extracellular domain of gp130 on one chain and the extracellular domain of IL-6Rα on the other chain. Like their Fc-based counterparts, such proteins were postulated to be high affinity ligand traps for IL-6 and as a result to inhibit the interaction of IL-6 with the native receptor on IL-6-responsive cells, thus functioning as IL-6 antagonists. Furthermore, constructs employing the full length Cγ region would, much like antibodies, form homodimers of the Cγ chain, giving rise to antibody-like molecules comprising of two "light chains" and two "heavy chains" (Figure 8). The potential advantage of this design is that it may more closely mimic the IL-6•IL-6Rα•gp130 complex and may display a higher affinity for the ligand than comparable single heterodimers. An additional design is incorporated by using truncated versions of Cγ, comprised only of the C_H1 domain. These will form heterodimeric molecules with receptor-κ fusion proteins, and will thus resemble the Fab fragment of antibodies.

All the soluble receptor-Ig chimeric genes may be engineered in plasmid vectors including, but not limited to, vectors suitable for mammalian expression (COS monkey kidney cells, Chinese Hamster Ovary cells [CHO], and ras-transformed fibroblasts [MG-ras]) and include a Kozak sequence (CGC CGC CAC CAT GGT G) at the beginning of each chimeric gene for efficient translation. Engineering was performed using standard genetic engineering methodology. Each construct was verified by DNA sequencing, mammalian expression followed by western blotting with suitable antibodies, biophysical assays that determine ligand binding and dissociation, and by growth inhibition assays (XG-1, as described later). Since the domains utilized to engineer these chimeric proteins are flanked by appropriate restriction sites, it is possible to use these domains to engineer other chimeric proteins, including chimeras employing the extracellular domains of the receptors for factors such as IL-1, IL-2, IL-3, IL-4, IL-5, GM-CSF, LIF, IL-11, IL-15, IFNγ, TGFβ, and others. The amino acid coordinates for each component utilized in making the IL-6 traps are listed below (Note: numbering starts with the initiating methionine as #1; long sequences are listed using the single letter code for the twenty amino acids):

(a) Constructs employing human gp130:

(i) **gp130-Cγ1** was engineered by fusing in frame the extracellular domain of gp130 (amino acids 1 to 619) to a Ser-Gly bridge, followed by the 330 amino acids which comprise Cγ1 and a termination codon (Figure 9).

(ii) **gp130-J-Cγ1** was engineered in the same manner as gp130-Cγ1 except that a J-peptide (amino acid sequence: GQGTLVTVSS) was inserted between the Ser-Gly bridge and the sequence of Cγ1 (see Figure 9).

(iii) **gp130Δ3fibro-Cγ1** was engineered by fusing in frame the extracellular domain of gp130 without its three fibronectin-like domains (Figure 10). The remaining part of this chimeric protein is identical to gp130-Cγ1.

(iv) **gp130-J-C_H1** was engineered in a manner identical for that described for gp130-Cγ1, except that in place of the Cγ1 region only the C_H1 part of Cγ1 has been used (Figure 11). The C-terminal domain of this construct includes the part of the hinge that contains the cysteine residue responsible for heterodimerization of the heavy chain of IgG with a light chain. The part of the hinge that contains the two cysteines involved in Cγ1 homodimerization has been deleted along with the C_H2 and C_H3 domains.

(v) **gp130-Cγ4** was engineered in a manner identical to that described for gp130-Cγ1, except that Cγ4 was used in place of Cγ1 (Figure 12). In addition, an *Rsr*II DNA restriction site was engineered at the hinge region of the Cγ4 domain by introducing two silent base mutations. The *Rsrs*II site allows for other desired genetic engineering manipulations, such as the construction of the C_H1 equivalent of gp130-Cγ4.

(vi) **gp130-κ** was engineered in a manner identical to that described for gp130-Cγ1, except that the constant region of the κ light chain of human Ig was used in place of Cγ1 (Figure 13).

(vi) **gp130-J-κ** was engineered in a manner identical to that described for gp130-J-κ, except that a j-peptide (amino acid sequence: TFGQGTKVEIK) was inserted between the Ser-Gly bridge and the κ-region.

(viii) **gp130-λ** was engineered in a manner identical to that described for gp130-Cγ1, except that the constant region of the λ light chain (Cheung, et al., Journal of Virology 66:6714-6720 (1992) of human Ig was used in place of Cγ1 (Figure 14).

(b) Constructs employing human IL-6Rα:

(i) **IL6Rα-Cγ1** was engineered by fusing in frame amino acids 1 to 358 of IL-6Rα (Yamasaki et al., Science 241:825-828 (1988), which comprise the

extracellular domain of IL-6Rα (Figure 15), to an Ala-Gly bridge, followed by the 330 amino acids which comprise Cγ1 and a termination codon.

(ii) **IL6Rα-κ** was engineered as described for IL6Rα-Cγ1, except that the κ-domain (Figure 13) utilized for gp130-κ was used in place of Cγ1.

(iii) **IL6Rα-j-κ** was engineered as described for IL6Rα-κ except that the j-peptide described for gp130-j-κ was placed between the Ala-Gly bridge and the κ-domain.

(iv) Three additional constructs, **IL6Rα313-Cγ1**, **IL6Rα313-κ**, and **IL6Rα313-j-κ**, were engineered as using a truncated form of IL-6Rα comprised of amino acids 1 to 313 (Figure 16). Each of these constructs were made by fusing in frame IL6Rα313 with a Thr-Gly bridge followed by the Cγ1, κ-, and j-κ-domains described above. These constructs were engineered in order to complement the gp130Δ3fibro-derived constructs.

Expression and purification of ligand traps

To produce covalently linked heterodimers of soluble gp130 and soluble IL-6Rα, gp130-Ig chimeric proteins were co-expressed with appropriate IL-6Rα-Ig chimeric proteins in complementing pairs. Co-expression was achieved by co-transfecting the corresponding expression vectors into suitable mammalian cell lines, either stably or transiently. The resulting disulfide-linked heterodimers were purified from conditioned media by several different methods, including but not limited to affinity chromatography on immobilized Protein A or Protein G, ligand-based affinity chromatography, ion exchange, and gel filtration.

An example of the type of methods used for purification of a heavy/light receptor fusion protein is as follows: gp130-Cγ1•IL-6Rα-κ was expressed in COS cells by co-transfecting two different vectors, encoding gp130-Cγ1 and

IL-6Rα-κ respectively. Serum-free conditioned media (400 ml) were collected two days post-transfection and Cγ1-bearing proteins were purified by affinity chromatography over a 1ml Protein A Sepharose (Pharmacia). The material generated in this step was further purified by a second affinity chromatography step over a 1 ml NHS-activated Sepharose (Pharmacia) which was derivatized with recombinant human IL-6, in order to remove gp130-Cγ1 dimer from gp130-Cγ1•IL-6Rα-κ complexes (the gp130-Cγ1 dimer does not bind IL-6). Proteins generated by this method were more than 90% pure, as evidenced by SDS-PAGE followed by silver-staining (Figure 17). Similar protocols have been employed successfully towards the purification of other heavy/light receptor heterodimers.

RESULTS

Biological activity of immunoglobulin heavy/light chain receptor fusion antagonists

The purified ligand traps were tested for their ability to bind IL-6 in a variety of different assays. For example, the dissociation rate of IL-6 bound to the ligand trap was measured in parallel with the dissociation rate of IL-6 from the anti-IL-6 monoclonal neutralizing antibody B-E8 [Brochier, et al., Int. J. Immunopharmacology 17:41-48 (1995), and references within]. An example of this type of experiment is shown in Figure 18. In this experiment 20 pM ^{125}I-IL-6 (1000 μCi/mmol; Amersham) was preincubated with 500 pM of either gp130-Cγ1•IL-6Rα-κ or mAb B-E8 for 20 hours. At this point a 1000-fold excess (20 nM) of "cold" IL-6 was added. Periodically, aliquots of the reaction were removed, the ligand trap or B-E8 were precipitated with Protein G-Sepharose, and the number of cpm of ^{125}I-IL-6 that remained bound was determined. Clearly, the dissociation rate of human ^{125}I-IL6 from the ligand trap was very slow - after three days, approximately 75% of the initial counts were still bound to the ligand

trap. In contrast, less than 5% of the counts remained associated with the antibody after three days. This result demonstrates that the dissociation rate of the ligand from these ligand traps is very slow.

In a different set of experiments the ability of the ligand traps to multimerize in the presence of ligand was tested. An example of this is shown in Figure 19. IL-6-induced association of gp130-Fc•IL-6Rα-Fc with gp130-C_H1•IL-6Rα-κ was determined by testing whether gp130-C_H1•IL-6Rα-κ, which does not by itself bind Protein A, could be precipitated by Protein A-Sepharose in the presence of gp130-Fc•IL-6Rα-Fc in an IL-6-depended manner (Figure 9). Precipitation of gp130-C_H1•IL-6Rα-κ by Protein A-Sepharose was determined by western blotting with an anti-kappa specific HRP conjugate, which does not detect gp130-Fc•IL-6Rα-Fc. gp130-C_H1•IL-6Rα-κ could be precipitated by Protein A-Sepharose only when both gp130-Fc•IL-6Rα-Fc and IL-6 were present. This result conclusively indicates that IL-6 can induce ligand trap multimerization, and further indicate that the ligand trap can mimic the hexameric cytokine•Rα•signal transducer complex (Figure 1). Ligand-induced multimerization may play a significant role in the clearance of cytokine•ligand trap complexes *in vivo*.

The biological activity of the different ligand traps may be further tested in assays which measure ligand-depended cell proliferation. Several cell proliferation assays exist for IL-6 and they employ cell lines such as B9, CESS, or XG-1. An example of this type of assay using the XG-1 cell line is presented below: XG-1 is a cell line derived from a human multiple myeloma (Zhang, et al., Blood 83:3654-3663 (1994). XG-1 depends on exogenously supplied human IL-6 for survival and proliferation. The EC_{50} of IL-6 for the XG-1 line is approximately 50 pmoles/ml. The ability of several different IL-6 traps to block IL-6-depended proliferation of XG-1

cells was tested by incubating increasing amounts of purified ligand traps with 50 pg/ml IL-6 in XG-1 cultures. The ligand traps which were tested had been expressed and purified by methods similar to those described above. All of the ligand traps tested were found to inhibit IL-6-dependent proliferation of XG-1 in a dose dependent manner (Figure 20). Of the five different traps tested gp130-Cγ1•IL-6Rα-κ was the most active and essentially display the same neutralizing activity towards IL-6 as the antibody B-E8. As little as a 10-fold molar excess of either gp130-Cγ1•IL-6Rα-κ or B-E8 completely blocked the activity of IL- 6 (a reading of A570-650 = 0.3 AU corresponds to no proliferation of the XG-1 cells). At a 100-fold molar excess all of the ligand traps tested completely blocked the activity of IL-6. This observed inhibition is highly selective as neither a gp130-Fc•CNTFRα-Fc ligand trap which blocks CNTF activity, nor gp130-Fc homodimer exhibit any blocking activity towards IL-6 even when used at a 1000-fold molar excess over IL-6 (data not shown). This data demonstrates that the heteromeric immunoglobulin heavy/light chain receptor-based ligand traps function as selective high affinity antagonists of their cognate ligand.

EXAMPLE 5 - CLONING OF FUSION POLYPEPTIDE COMPONENTS

The extracellular domains of the human cytokine receptors were obtained by standard PCR techniques using tissue cDNAs (CLONTECH), cloned into the expression vector, pMT21 (Genetics Institute, Inc.), and the sequences were sequenced by standard techniques using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). For the IL-4Rα, nucleotides 241 through 868 (corresponding to the amino acids 24-231) from the Genbank sequence, X52425, were cloned. For the IL-2Rγ, nucleotides 15 through 776 (corresponding to amino acids 1-233) from the Genbank sequence, D11086, were cloned. For the IL-6Rα, nucleotides 52 through 1044 (corresponding

to the amino acids 1-331) from the Genbank sequence, X52425, were cloned. For gp130, nucleotides 322 through 2112 (corresponding to the amino acids 30-619) from the Genbank sequence, M57230, were cloned. For the IL-1RAcP, nucleotides 1 through 1074 (corresponding to the amino acids 1-358) from the Genbank sequence, AB006357, were cloned. For the IL-1RI, nucleotides 55 through 999 (corresponding to the amino acids 19-333) from the Genbank sequence, X16896, were cloned.

EXAMPLE 6 - PRODUCTION OF FUSION POLYPEPTIDES (CYTOKINE TRAPS)

The nucleotide sequences encoding the cytokine traps were constructed from the individual cloned DNAs (described *supra*) by standard cloning and PCR techniques. In each case, the sequences were constructed in frame such that the sequence encoding the first fusion polypeptide component was fused to the sequence encoding the second fusion polypeptide component followed by an Fc domain (hinge, CH2 and CH3 region of human IgG1) as the multimerizing component. In some cases extra nucleotides were inserted in frame between sequences encoding the first and second fusion polypeptide components to add a linker region between the two components (See Figure 21A - Figure 21D - trap 424; Figure 24A - Figure 24F - trap 412; and Figure 26A - Figure 26E - trap 569).

For the IL-4 traps, 424 (Figure 21A - Figure 21D), 603 (Figure 22A - Figure 22D) and 622 (Figure 23A - Figure 23D), the IL-2Rγ component is 5′, followed by the IL4Rα component and then the Fc component. For the IL-6 traps, 412 (Figure 24A - Figure 24F) and 616 (Figure 25A - Figure 25F), the IL-6Rα component is 5′ followed by the gp130 component and then the Fc domain. For the IL-1 trap 569 (Figure 26A - Figure 26E), the IL-1RAcP component is 5′ followed by the IL-1RI component and then the Fc domain. The final constructs were cloned into the mammalian expression vector pCDNA3.1 (STRATAGENE).

In the 569 sequence (Figure 26A - Figure 26E), nucleotides 1-1074 encode the IL1RAcP component, nucleotides 1075 -1098 encode a linker region, nucleotides 1099-2043 encode the IL1RI component and nucleotides 2044-2730 encode the Fc domain.

In the 412 sequence (Figure 24A - Figure 24F), nucleotides 1-993 encode the IL6Rα component, nucleotides 994-1023 encode a linker region, nucleotides 1024-2814 encode the gp130 component and nucleotides 2815-3504 encode the Fc domain.

In the 616 sequence (Figure 25A - Figure 25F), nucleotides 1-993 encode the IL6Rα component, nucleotides 994-2784 encode the gp130 component and nucleotides 2785-3474 encode the Fc domain.

In the 424 (Figure 21A - Figure 21D) and 622 (Figure 23A - Figure 23D) sequences, nucleotides 1-762 encode the IL2Rγ component, nucleotides 763-771 encode a linker region, nucleotides 772-1395 encode the IL4Rα component and nucleotides 1396-2082 encode the Fc domain.

Finally, in the 603 sequence (Figure 22A - Figure 22D), nucleotides 1-762 encode the IL2Rγ component, nucleotides 763-1386 encode the IL4Rα component and nucleotides 1387-2073 encode the Fc domain.

DNA constructs were either transiently transfected into COS cells or stably transfected into CHO cells by standard techniques well known to one of skill in the art. Supernatants were collected and purified by Protein A affinity chromatography and size exclusion chromatography by standard techniques. (See for example Harlow and Lane, Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory, 1988).

EXAMPLE 7: IL-4 BIOASSAY PROTOCOL USING TF-1 (ATCC) CELLS.

Reagents and Equipment Needed

MTT Dye Solution:

MTT(3-[4,5-Dimethylthiazole-2-yl]) (Sigma catalog# M2128)
Working concentration: Dissolve 5 mg of anhydrous MTT in 200 ml PBS without Ca^{+2}, Mg^{+2}.
Sterile filter and store aliquoted at -20°C

Solubilization Solution:

For 1000 ml, combine 100 g SDS, 950 ml dH_20, 50 ml Dimethyl Formamide, and 850 µl concentrated HCl.
Filter sterilize with a 0.45µm filter unit.
Store at room temperature

TF-1 cell Growth Medium:

RPMI 1640, 10% FBS, Pen/Strep, 2mM L-glutamine

Other:

0.4% Trypan Blue Stain, sterile tubes for dilutions, sterile 96 well cell culture plates (Falcon #3072), hemacytometer, centrifuge, ELISA plate reader, multichannel pipet for 15, 25, 50 and 100µl volume, sterile reagent reservoirs, sterile pipet tips, gloves.

Assay Protocol

A. Preparation of Assay plates

1. Prepare sterile 96 well tissue culture plates to contain 50µl of growth medium per well with various concentrations of IL-4 and 10nM IL-4 antagonist. This can be done by preparing a working dilution of IL-4 that is 4 times the highest concentration to be assayed. In separate tubes, do a two-fold serial dilution of the IL-4. Add 25µl of each dilution to one row across the plate (i.e. row A gets highest concentration, row G gets lowest concentration). Add 25µl of growth medium without IL-4 to row H. Prepare the antagonists to be tested by making a stock that is 4 times the final concentration. Add 25µl to a triplicate set of IL-4 containing wells (columns 1,2,3, A through H). Be sure to include antagonist in row H.

2. As a positive control, leave one set with no antagonist. These wells will contain IL-4 and media only.

3. Incubate the plate for 1-2 hours at 37°C in a humidified 5% CO_2 incubator before preparing cells to be used for assay.

B. Preparation of Cells

4. Wash cells twice by centrifugation in assay medium free of growth factor.

5. Determine cell number and trypan blue viability and suspend cells to a final concentration of 8×10^5/ml in assay medium.

6. Dispense 50µl of the cell suspension (40,000 cells) into all wells of the plates. Total volume should now be 100µl/well.

7. Incubate the plate at 37°C for 68 hours in a humidified 5% CO_2 incubator.

C. Color Development

8. After incubating for 68 hours, add 15µl of the MTT dye solution to each well.

9. Incubate the plate at 37°C for 4 hours in a humidified 5% CO_2 incubator.

10. After 4 hours, add 100µl of the solubilization solution to each well. Allow the plate to stand overnight in a sealed container to completely solubilize the formazan crystals.

11. Record the absorbance at 570/650nm.

RESULTS

Figure 27 shows that an IL-4 trap designated 4SC375, which is a fusion polypeptide of IL-2Rγ-scb-IL4Rα-FcΔC1, is several orders of magnitude better as an IL-4 antagonist than IL4RαFcΔC1 alone in the TF1 cell bioassay.

Figure 28 shows that the IL-4 trap designated 4SC375 shows antagonistic activity in the TF1 cell bioassay equivalent to an IL-4 trap designated 4SC424 which is a fusion polypeptide of IL-2Rγ-IL4Rα-FcΔC1 having the IL-2Rγ component flush with the IL-4Rα component.

EXAMPLE 8: IL-6 BIOASSAY PROTOCOL USING XG-1 CELLS

Reagents and Equipment Needed

MTT Dye Solution:

MTT(3-[4,5-Dimethylthiazole-2-yl]) (Sigma catalog# M2128)
Working concentration: Dissolve 5 mg of anhydrous MTT in 200 ml PBS without Ca^{+2}, Mg^{+2}.
Sterile filter and store aliquoted at -20°C

Solubilization Solution:

For 1000 ml, combine 100 g SDS, 950 ml dH_20, 50 ml Dimethyl Formamide, and 850 µl concentrated HCl.
Filter sterilize with at 0.45µm filter unit.
Store at room temperature

Assay Medium:

RPMI 1640, 10%FBS, Pen/Strep, 2mM L-glutamine, 50µM mercapto-ethanol.

Other:

0.4% Trypan Blue Stain, sterile tubes for dilutions, sterile 96 well cell culture plates (Falcon#3072), hemacytometer, centrifuge, ELISA plate reader, multichannel pipet for 15, 25, 50 and 100µl volume, sterile reagent reservoirs, sterile pipet tips, gloves.

Assay Protocol

A. Preparation of Assay plates

1. Prepare sterile 96 well tissue culture plates to contain 50µl of growth medium per well with various concentrations of IL-6 and 10nM IL-6 antagonist. This can be done by preparing a working dilution of IL-6 that is

4 times the highest concentration to be assayed. In separate tubes, do a two-fold serial dilution of the IL-6. Add 25µl of each dilution to one row across the plate (i.e. row A gets highest concentration, row G gets lowest concentration). Add 25µl of growth medium without IL-6 to row H. Prepare the antagonists to be tested by making a stock that is 4 times the final concentration. Add 25µl to a triplicate set of IL-6 containing wells (columns 1,2,3, A through H). Be sure to include antagonist in row H. A typical IL-6 titration starts at 200ng/ml down to 3.1ng/ml.

2. As a positive control, leave one set with no antagonist. These wells contain IL-6 and media in place of antagonist.

3. Incubate the plate 1-2 hours at 37oC in a humidified 5% CO_2 incubator before preparing cells to be used for assay.

B. Preparation of Cells

4. Wash cells twice by centrifugation (5 min at 1000RPM) in assay medium free of growth factor.

5. Determine cell number and trypan blue viability and suspend cells to a final concentration of 8 x 10^5/ml in assay medium.

6. Dispense 50µl of the cell suspension (40000 cells) into all wells of the plates. Total volume should now be 100µl/well.

7. Incubate the plate at 37°C for 68 hours in a humidified 5% CO_2 incubator.

C. Color Development

8. At 68 hours add 15µl of the dye solution to each well.

9. Incubate the plate at 37°C for 4 hours in a humidified 5% CO_2 incubator.

10. After 4 hours, add 100μl of the solubilization solution to each well. Allow the plate to stand overnight in a sealed container to completely solubilize the formazan crystals.

11. Record the absorbance at 570/650nm.

RESULTS

Figure 29 shows that the IL6 trap (6SC412 IL6R-scb-gpx-FcΔC1) described in Figure 24A - Figure 24F is a better antagonist of IL-6 in the XG1 bioassay than the neutralizing monoclonal antibody to human IL-6 - BE8.

EXAMPLE 9: MRC5 BIOASSAY FOR IL1 TRAPS

MRC5 human lung fibroblast cells respond to IL-1 by secreting IL-6 and thus were utilized to assay the ability of IL-1 traps to block the IL-1-dependent production of IL-6. IL1 Trap 1SC569 (Figure 26A - Figure 26E) was tested against IL-1-RI.Fc which is the extracellular domain of the IL-1 Type I receptor fused to an Fc domain.

MRC5 cells are suspended at 1 x 10^5 cells per ml in medium and 0.1 ml of cells are plated (10,000 cells per well) into the wells of a 96 well tissue culture plate. Plates are incubated for 24 hours at 37°C in a humidified 5% CO_2 incubator.

IL-1 trap and recombinant human IL-1 at varying doses are pre-incubated in a 96 well tissue culture dish and incubated for 2 hours at 37°C. 0.1 ml of this mixture is then added to the 96 well plate containing the MRC5 cells such that the final concentration of IL-1 Trap is 10nM and the final

concentrations of the IL-1 ranges from 2.4 pM to 5nM. Control wells contain trap alone or nothing.

Plates are then incubated at 37°C for 24 hours in a humidified 5% CO_2 incubator. Supernatant is collected and assayed for levels of IL-6 using R&D Systems Quantikine Immunoassay Kit according to the manufacturer's instructions.

RESULTS

Figure 30 shows that the trap 569 (Figure 26A - Figure 26E) is able to antagonize the effects of IL-1 and block the IL-6 production from MRC 5 cells upon treatment with IL-1. At a concentration of 10nM, the trap 569 is able to block the production of IL-6 up to an IL-1 concentration of 3nM. In contrast, the IL-1RI.Fc is a much poorer antagonist of IL-1. It is only able to block the effects of IL-1 up to about 10-20 pM. Thus, the trap 569 is approximately 100x better at blocking IL-1 than IL1RI.Fc.

EXAMPLE 10 - CONSTRUCTION OF IL-13/IL-4 SINGLE CHAIN TRAPS

1. To create the IL-13/IL-4 dual trap designated IL-4Rα.IL-13Rα1.Fc, the human IL-4Rα extracellular domain (corresponding to nucleotides #1-693 of Figure 31A - Figure 31G) and the human IL-13Rα1 extracellular domain (corresponding to nucleotides #700-1665 of Figure 31A - Figure 31G) were amplified by standard PCR techniques and ligated into an expression vector pMT21 which contained the human Fc sequence (corresponding to nucleotides #1671-2355 of Figure 31A - Figure 31G), thus creating a fusion protein consisting of the IL-4Rα , IL-13Rα1, and the hinge, CH2 and CH3 region of human IgG1 from the N to C terminus. In addition, a two amino acid linker (corresponding to nucleotides #694-699 of Figure 31A - Figure 31G) with the amino acid sequence SerGly was constructed in frame

between the IL-4Rα and the IL-13Rα1 and a two amino acid linker (corresponding to nucleotides #1666-1671 of Figure 31A - Figure 31G) with the amino acid sequence ThrGly was constructed in frame between the IL-13Rα1 and the Fc portion. All sequences were sequence-verified by standard techniques. The IL-4Rα.IL-13Rα1.Fc coding sequence was then subcloned into the expression vector pCDNA3.1 (Stratagene) using standard molecular biology techniques.

2. To create the IL-13/IL-4 dual trap designated IL-13Rα1.IL-4Rα.Fc, the IL-13Rα1 extracellular domain (corresponding to nucleotides #1-1029 of Figure 32A - Figure 32G) and the human IL-4Rα (corresponding to nucleotides # 1060-1692 of Figure 32A - Figure 32G) were amplified by standard PCR techniques and ligated into the expression vector pJFE14, which contains the human Fc sequence (corresponding to nucleotides #1699-2382 of Figure 32A - Figure 32G) to create a fusion protein consisting of the IL-13Rα1, IL-4Rα, and the hinge, CH2 and CH3 region of human IgG1 from the N to C terminus. In addition, a ten amino acid linker with the amino acid sequence GlyAlaProSerGlyGlyGlyGlyArgPro (corresponding to nucleotide #1030-1059 of Figure 32A - Figure 32G) was constructed in frame between the IL-13Rα1 and the IL-4Rα and a two amino acid linker (corresponding to nucleotides #1693-1698 of Figure 32A - Figure 32G) with the amino acid sequence SerGly was constructed in frame between IL-4Rα and the Fc portion. All sequences were sequence-verified using standard techniques. The coding sequence of IL-13Rα1.IL-4Rα.Fc was then subcloned into the expression vector pCDNA3.1 (Stratagene) using standard molecular biology techniques.

EXAMPLE 11: EXPRESSION OF IL-4Rα.IL-13Rα1.Fc AND IL-13Rα1.IL-4Rα.Fc

Large scale (1L) cultures of the pCAE801 (the DNA vector construct encoding IL-4Rα.IL-13Rα1.Fc) and pCAE802 (the DNA plasmid construct encoding IL-13Rα1.IL-4Rα.Fc) in DH10B cells were grown overnight in LB + ampicillin and the plasmid DNA was extracted using a Qiagen Endofree Mega Kit following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined in a UV spectrophotometer and fluorometer. The plasmid DNA was also verified by digestion of aliquots with BbsI, XmnI and NcoI restriction enzymes. All restriction enzyme digest fragments corresponded to the predicted sizes in a 1% agarose gel.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4×10^6 cells/plate. Plating media was Gibco Ham's F-12 w/10% Hyclone Fetal Bovine Serum (FBS) + penicillin/streptomycin and supplemented with glutamine. The following day each plate was transfected with 6 μg of pCAE801, or pCAE802, using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells 12 ml/plate of Optimem w/ 10% FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II w/ glutamine + 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days.

After 3 days of incubation the media was removed from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1L bottles and expressed protein was purified as described *infra*.

EXAMPLE 12: PURIFICATION OF IL-4Rα.IL-13Rα1.Fc AND IL-13Rα1.IL-4Rα.Fc PROTEIN FROM CULTURE MEDIA

1. Purification of IL-4Rα.IL-13Rα1.Fc.

Human IL-4Rα.IL-13Rα1.Fc was transiently expressed in CHO cells and supernatants were harvested from plate transfections as described *supra.* Expression of the secreted protein was determined by a sandwich ELISA using goat anti-hIgG (γ chain specific; Sigma 1-3382) and goat anti-hIgG (Fc specific)-FITC conjugate (Sigma F9512) capture and report antibodies, respectively. The yield ranged from 5.8 to 9.2 mg (average of 7.5 mg) per liter of conditioned media. Complete™ protease inhibitor tablets (Roche Diagnostics Corp.) were dissolved into the media (1 tablet/L). The conditioned media was sterile filtered (0.22 μm pore size) prior to loading onto a pre-equilibrated, 5 mL HiTrap® Protein A affinity column (Amersham Pharmacia Biotech) in Dulbecco's PBS buffer (Life Technologies), pH 7.4 at 4°C. The flow rate was ~1-2 mL/min. The column was extensively washed with PBS buffer to remove nonspecifically bound proteins from the column. IL-4Rα.IL-13Rα1.Fc was eluted using 20 mM sodium citrate, 150 mM NaCl, pH 3.5. The eluate was immediately neutralized by titrating with 1 M Tris-OH. The fractions containing protein were pooled and immediately dialyzed in PBS buffer, pH 7.4 at 4°C. The recovery from Protein A purification was 6.8 mg (73%). IL-4Rα.IL-13Rα1.Fc was further purified by size exclusion chromatography using a superose 6 column (25 mL bed volume; Amersham Pharmacia Biotech) pre-equilibrated in PBS, 5% v/v glycerol, pH 7.4 at ambient temperature. The flow rate was 0.5 mL/min. Protein fractions were assessed from a Coomassie stained non-reduced and reduced SDS-PAGE (Novex NuPAGE 4-12% Bis-Tris gels). Fractions were conservatively pooled to reduce the amount of aggregated protein. The overall yield was 51% (4.4 mg) with a purity of 97% as judged by SDS-PAGE. Purified IL-4Rα.IL-13Rα1.Fc was analyzed by non-reduced and reduced SDS-PAGE (4-12% Bis-Tris), analytical size exclusion chromatography (Tosohaas

TSKG4000SWXL), N-terminal sequencing, and immunoblotting with goat anti-hIgG-HRP conjugate (Promega W403B), and also mouse monoclonal anti-hIL-4R (R&D MAB230) followed by anti-mIgG-HRP conjugate (Promega W402B) as the secondary antibody.

2. Purification of IL-13Rα1.IL-4Rα.Fc

Human IL-13Rα1.IL-4Rα.Fc was transiently expressed in CHO cells and supernatants were harvested from plate transfections as described *supra*. Expression of the secreted protein was determined by a sandwich ELISA using goat anti-hIgG (γ chain specific; Sigma 1-3382) and goat anti-hIgG (Fc specific)-FITC conjugate (Sigma F9512) capture and report antibodies, respectively. The yield was 8.8 mg per liter of conditioned media. Complete™ protease inhibitor tablets (Roche Diagnostics Corp.) were dissolved into the media (1 tablet/L). The conditioned media was sterile filtered (0.22 μm pore size) prior to loading onto a pre-equilibrated, 5 mL HiTrap® Protein A affinity column (Amersham Pharmacia Biotech) in Dulbecco's PBS buffer (Life Technologies), pH 7.4 at 4°C. The flow rate was ~1-2 mL/min. The column was extensively washed with PBS buffer to remove nonspecifically bound proteins from the column. IL-13Rα1.IL-4Rα.Fc was eluted using 20 mM sodium citrate, 150 mM NaCl, pH 3.5. The eluate was immediately neutralized by titrating with 1 M Tris-OH. The fractions containing protein were pooled and immediately dialyzed in PBS buffer, pH 7.4 at 4 °C. The recovery from Protein A purification was 3.8 mg (43%). IL-13Rα1.IL-4Rα.Fc was further purified by size exclusion chromatography using a superose 6 column (25 mL bed volume; Amersham Pharmacia Biotech) pre-equilibrated in PBS, 5% v/v glycerol, pH 7.4 at ambient temperature. The flow rate was 0.5 mL/min. Protein fractions were assessed from a Coomassie stained non-reduced and reduced SDS-PAGE (Novex NuPAGE 4-12% Bis-Tris gels). Fractions were

conservatively pooled to reduce the amount of aggregated protein. The overall yield was 17% (1.5 mg) with a purity of 95% as judged by SDS-PAGE. Purified IL-13Rα1.IL-4Rα.Fc was analyzed by non-reduced and reduced SDS-PAGE (4-12% Bis-Tris), analytical size exclusion chromatography (Tosohaas TSKG4000SWXL), N-terminal sequencing, and immunoblotting with goat anti-hIgG-HRP conjugate (Promega W403B), and also mouse monoclonal anti-hIL-4Rα (R&D MAB230) followed by anti-mIgG-HRP conjugate (Promega W402B) as the secondary antibody.

EXAMPLE 13: BLOCKING OF IL-4 AND IL-13 BY IL-4Rα.IL-13Rα1.Fc AND IL-13Rα1.IL-4Rα.Fc

Materials and Methods

TF1 Bioassay. TF1 cells were maintained in growth media (10ng/ml GM-CSF, RPMI 1640, 10% FBS, L-glutamine, Penicillin, Streptomycin). For the bioassay, cells were washed 2 times in assay media (as above but without GM-CSF) and then plated at 2 x 10^5 cells in 50µl of assay media. The purified IL-4Rα.IL-13Rα1.Fc and IL-13Rα1.IL-4Rα.Fc proteins were diluted into assay media at a concentration of 40nM. 25ul of each of the traps was added to the cells. Either IL-13 or IL-4 were diluted to 40nM in assay media and then 2-fold dilution series in assay media were made. 25µl of either IL-13 or IL-4 was then added to the wells containing the cells and the traps. Cells were then incubated at 37°C, 5% CO_2 for ~70 hrs. The extent of TF1 cell proliferation was measured by the MTS assay according to the manufacturer's protocol (Promega, Inc.).

RESULTS

The ability of the IL-4Rα.IL-13Rα1.Fc and IL-13Rα1.IL-4Rα.Fc traps to block both human IL-13 and human IL-4 activity was measured in the TF1

bioassay described *supra*. IL-13 stimulates proliferation of TF1 cells, with half-maximal growth at a concentration of 0.2nM. Addition of either IL-4Rα.IL-13Rα1.Fc or IL-13Rα1.IL-4Rα.Fc trap at a concentration of 10nM blocks IL-13-induced growth up to ~2nM (Figure 33). At an IL-13 concentration of ~4-5 nM the growth of TF1 cells is inhibited by 50%. TF1 cells are more sensitive to IL-4, which stimulates their proliferation with half-maximal growth at ~0.02nM. Addition of either IL-4Rα.IL-13Rα1.Fc or IL-13Rα1.IL-4Rα.Fc at a concentration of 10nM blocks IL-4-induced growth up to ~1nM (Figure 34). At an IL-4 concentration of ~3-4 nM the growth of TF1 cells is inhibited by 50%. These results show that both IL-4Rα.IL-13Rα1.Fc and IL-13Rα1.IL-4Rα.Fc can block the ability of both IL-13 and IL-4 to stimulate cellular responses.

EXAMPLE 14: BLOCKING OF INJECTED IL-1 BY IL-1 TRAP *IN VIVO*

IL-1 is a pro-inflammatory cytokine. Systemic administration of IL-1 has been shown to elicit acute responses in animals, including transient hyperglycemia, hypoinsulinemia, fever, anorexia, and increased serum levels of interleukin-6 (IL-6) (Reimers, 1998). Since mice are responsive to both murine and human IL-1, human IL-1 can be used and *in vivo* binding effects of human specific IL-1 antagonists can be evaluated. This acute mouse model was used to determine the ability of a human IL-1 trap to antagonize the *in vivo* effects of exogenously administered human IL-1. This provides a rapid indication of *in vivo* efficacy of the human IL-1 trap and can be used as an assay to help molecule selection.

Experimental Design:

Mice were given subcutaneous injections of human IL-1 (0.3 μg/kg). Twenty-four hours prior to human IL-1 injection, the animals were pre-treated with either vehicle or 150-fold molar excess of human IL-1 trap (0.54 mg/kg). Two hours prior to sacrifice (26 hrs), the mice were given a

second injection of human IL-1 (0.3 μg/kg). Blood samples were collected at various time points and sera were assayed for IL-6 levels.

RESULTS

Exogenous administration of human IL-1 resulted a dramatic induction of serum IL-6 levels. At 150-fold molar excess, the human IL-1 trap completely blocked the IL-6 increase (Figure 35). Furthermore, the effects of the human IL-1 trap persisted for at least another 24 hours, preventing an IL-6 increase even when IL-1 was re-administered (Figure 35). Such long-lasting efficacy suggests that daily injection of an IL-1 trap may not be necessary for chronic applications.

EXAMPLE 15: EVALUATING THE ABILITY OF AN IL-4 TRAP TO BLOCK THE PHYSIOLOGICAL RESPONSES TO HUMAN IL-4 IN CYNOMOLOGUS MONKEYS.

Systemic administration of human IL-4 elicits systemic responses in Cynomologus monkeys (Gundel et al., 1996). Thus, the effectiveness of the IL-4 trap in blocking human IL-4 can be demonstrated by measuring these responses.

Experimental Design:

The experiment consisted of 3 parts: human IL-4 + vehicle (part 1), human IL-4 + IL-4 Trap (part 2), and human IL-4 + vehicle (part 3). Human IL-4 (25 μg/kg) was injected subcutaneously twice daily for 4 days and IL-4 Trap (8 mg/kg) and vehicle were given intravenously daily for 5 days, beginning 1 day prior to human IL-4 administration. Whole blood was collected daily for flow cytometry analysis for CD16 and plasma was obtained to assay for the cytokine monocyte chemotactic protein 1 (MCP-1).

CD16 and MCP-1 are markers of IL-4-mediated inflammation in both humans and monkeys.

RESULTS

In the presence of human IL-4, MCP-1 increased 2.5-fold and was significantly blocked by the IL-4 Trap (Figure 36A). Similarly, the decrease in the percent of CD16 positive lymphocytes in peripheral blood was attenuated by the IL-4 trap (Figure 36B). After a rest period, the monkeys were re-injected with human IL-4 and the responsiveness of the animals to human IL-4 was re-confirmed (Figures 36A and 36B), suggesting that inhibition of the MCP-1 and CD 16 responses is specifically mediated by the IL-4 trap.

EXAMPLE 16: THE EFFECTS OF IL-4 TRAP ON 1L-4-INDUCED IgE SECRETION.

It has been shown that injection of anti-mouse IgD antibody stimulates an IL-4-mediated IgE increase in normal mice. This model has been widely used to evaluate IL-4 antagonists, such as soluble IL-4 receptor and anti-IL-4 monoclonal antibodies (Sato et al., 1993). We decided to use this model to evaluate the ability if the IL-4 trap to block IL-4-mediated increases of IgE.

Experimental design:

BALB/C mice injected with anti-mouse IgD (100μl/mouse, s.c.) were randomly divided into 3 groups. Each received (on days 3-5) either vehicle, murine IL-4 trap (1 mg/kg, s.c.), or a monoclonal antibody to mouse IL-4 (1 mg/kg, s.c.). Serum was collected at various time points and assayed for IgE levels.

RESULTS

Treatment with the murine IL-4 trap or the mouse IL-4 antibody both significantly antagonized the IL-4-mediated IgE increase in this mouse model (Figure 37). This suggests that the murine IL-4 trap binds murine IL-4 and antagonizes physiological responses elicited by endogenous IL-4 *in vivo*.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

WE CLAIM:

1. An isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex comprising:

a) a nucleotide sequence encoding a first fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the specificity determining component of the cytokine's receptor;

b) a nucleotide sequence encoding a second fusion polypeptide component comprising the amino acid sequence of the cytokine binding portion of the extracellular domain of the signal transducing component of the cytokine's receptor; and

c) a nucleotide sequence encoding a third fusion polypeptide component comprising the amino acid sequence of a multimerizing component.

2. The nucleic acid molecule of claim 1, wherein the nucleotide sequence encoding the first component is upstream of the nucleotide sequence encoding the second component.

3. The nucleic acid molecule of claim 1, wherein the nucleotide sequence encoding the first component is downstream of the nucleotide sequence encoding the second component.

4. The isolated nucleic acid molecule of claim 1, wherein the cytokine receptor is the receptor for a member of the hematopoietin family of cytokines selected from the group consisting of interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-9, interleukin-11, interleukin-13, interleukin-15, granulocyte macrophage colony stimulating factor, oncostatin M, and leukemia inhibitory factor and cardiotrophin-1

5. The isolated nucleic acid molecule of claim 1, wherein the cytokine receptor is the receptor for a member of the interferon family of cytokines selected from the group consisting of IFN-gamma, IFN-alpha, and IFN-beta.

6. The isolated nucleic acid molecule of claim 1, wherein the cytokine receptor is the receptor for a member of the immunoglobulin superfamily of cytokines selected from the group consisting of B7.1 (CD80) and B7.2 (B70).

7. The isolated nucleic acid molecule of claim 1, wherein the cytokine receptor is the receptor for a member of the TNF family of cytokines selected from the group consisting of TNF-alpha, TNF-beta, LT-beta, CD40 ligand, Fas ligand, CD 27 ligand, CD 30 ligand, and 4-1BBL.

8. The isolated nucleic acid molecule of claim 1, wherein the cytokine receptor is the receptor for a member of the TGF-β/BMP family selected from the group consisting of TGF-β1, TGF-β2, TGF-β3, BMP-2, BMP-3a, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8a, BMP-8b, BMP-9, BMP-10, BMP-11, BMP-15, BMP-16, endometrial bleeding associated factor (EBAF), growth differentiation factor-1 (GDF-1), GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-12, GDF-14, mullerian inhibiting substance (MIS), activin-1, activin-2, activin-3, activin-4, and activin-5.

9. The isolated nucleic acid molecule of claim 1, wherein the cytokine receptor is the receptor for a cytokine selected from the group consisting of interleukin-1, interleukin-10, interleukin-12, interleukin-14, interleukin-18 and MIF.

10. The isolated nucleic acid molecule of claim 1, wherein the multimerizing component comprises an immunoglobulin derived domain.

11. The isolated nucleic acid molecule of claim 10, wherein the immunoglobulin derived domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

12. A fusion polypeptide encoded by the isolated nucleic acid molecule of claim 1.

13. A composition capable of binding a cytokine to form a nonfunctional complex comprising a multimer of the fusion polypeptide of claim 12.

14. The composition of claim 13, wherein the multimer is a dimer.

15. A vector which comprises the nucleic acid molecule of claim 1.

16. An expression vector comprising a nucleic acid molecule of claim 1, wherein the nucleic acid molecule is operatively linked to an expression control sequence.

17. A host-vector system for the production of a fusion polypeptide which comprises the expression vector of claim 16, in a suitable host cell.

18. The host-vector system of claim 17, wherein the suitable host cell is a bacterial cell, yeast cell, insect cell, or mammalian cell.

19. The host-vector system of claim 17, wherein the suitable host cell is E. coli.

20. The host-vector system of claim 17, wherein the suitable host cell is a COS cell.

21. The host-vector system of claim 17, wherein the suitable host cell is a CHO cell.

22. The host-vector system of claim 17, wherein the suitable host cell is a 293 cell.

23. The host-vector system of claim 17, wherein the suitable host cell is a BHK cell.

24. The host-vector system of claim 17, wherein the suitable host cell is a NS0 cell.

25. A method of producing a fusion polypeptide which comprises growing cells of the host-vector system of claim 17, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

FIGURE 1

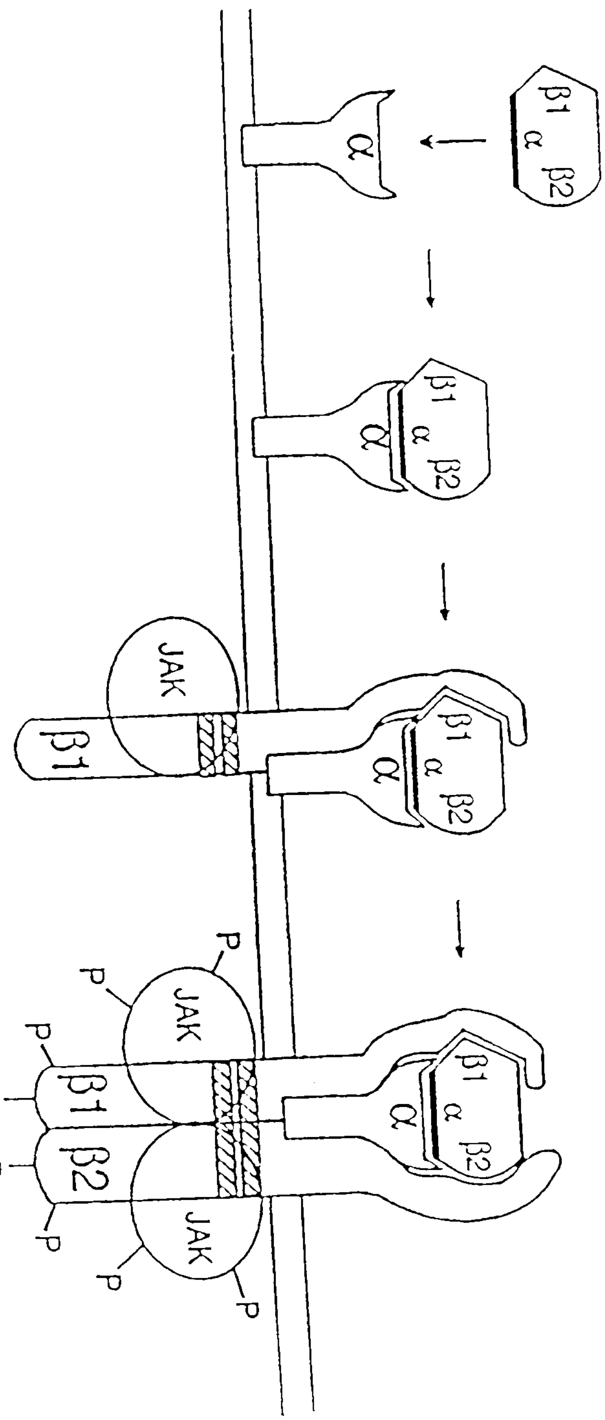


FIGURE 2

FIGURE 3

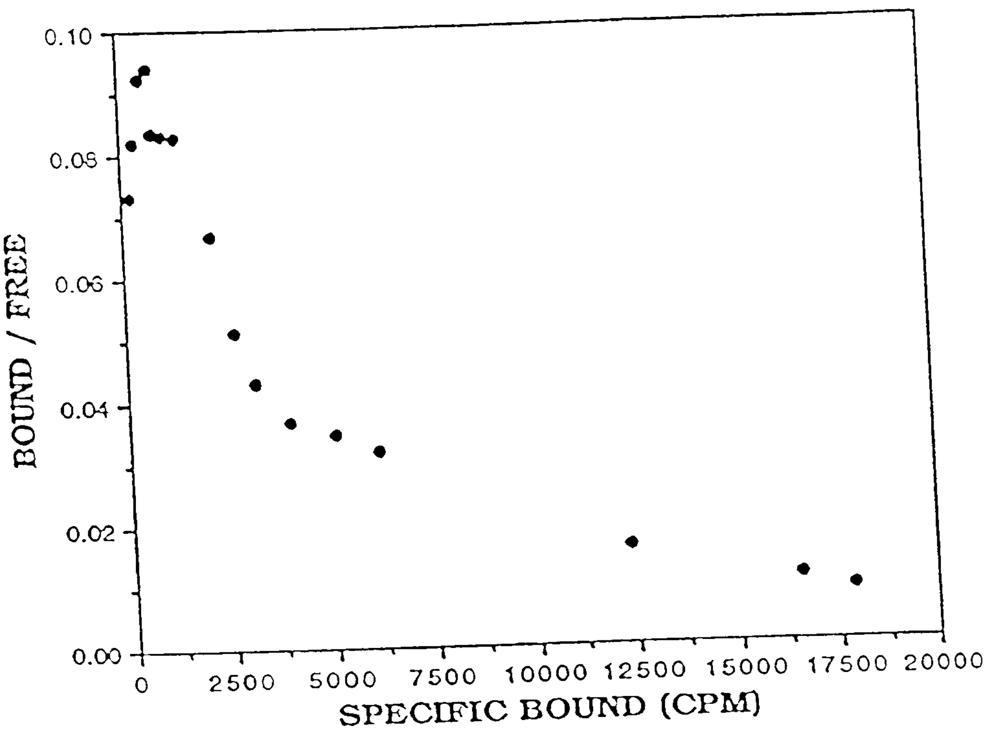

BOUND / FREE
0.10
0.08
0.06
0.04
0.02
0.00
0
2500
5000
7500
10000
12500
15000
17500
20000
SPECIFIC BOUND (CPM)

Figure 4

Amino acid sequence of human gp130-Fc-His6

Sequence Range: 1 to 861

10 20 30 40 50 60

MVTLQTWVVQALFIFLTTES TGELLDPCGYISPESPVVQL HSNFTAVCVLKEKCMDYFHV

70 80 90 100 110 120

NANYIVWKTNHFTIPKEQYT IINRTASSVTFTDIASLNIQ LTCNILTFGQLEQNVYGITI

130 140 150 160 170 180

ISGLPPEKPKNLSCIVNEGK KMRCEWDGGRETHLETNFTL KSEWATHKFADCKAKRDTPT

190 200 210 220 230 240

SCTVDYSTVYFVNIEVWVEA ENALGKVTSDHINFDPVYKV KPNPPHNLSVINSEELSSIL

250 260 270 280 290 300

KLTWTNPSIKSVIILKYNIQ YRTKDASTWSQIPPEDTAST RSSFTVQDLKPFTEYVFRIR

310 320 330 340 350 360

CMKEDGKGYWSDWSEEASGI TYEDRPSKAPSFWYKIDPSH TQGYRTVQLVWKTLPPFEAN

370 380 390 400 410 420

GKILDYEVTLTRWKSHLQNY TVNATKLTVNLTNDRYLATL TVRNLVGKSDAAVLTIPACD

430 440 450 460 470 480

FQATHPVMDLKAFPKDNMLW VEWTTPRESVKKYILEWCVL SDKAPCITDWQQEDGTVHRT

490 500 510 520 530 540

YLRGNLAESKCYLITVTPVY ADGPGSPESIKAYLKQAPPS KGPTVRTKKVGKNEAVLEWD

550 560 570 580 590 600

QLPVDVQNGFIRNYTIFYRT IIGNETAVNVDSSHTEYTLS SLTSDTLYMVRMAAYTDEGG

610 620 630 † † 640 650 660

KDGPEFTFTTPKFAQGEIES GEPKSCDKTHTCPPCPAPEL LGGPSVFLFPPKPKDTLMIS

670 680 690 700 710 720

RTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREE QYNSTYRVVSVLTVLHQDWL

730 740 750 760 770 780

FIGURE 4 continued

<u>NGKEYKCKVSNKALPAPIEK TISKAKGQPREPQVYTLPPS RDELTKNQVSLTCLVKGFYP</u>

```
        790        800          810        820          830        840
          *          *            *          *            *          *
```

<u>SDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDK SRWQQGNVFSCSVMHEALHN</u>

```
        850        860
          *          *
```

<u>HYTQKSLSLSPGK</u>*HHHHHH*•

FIGURE 5

The amino acid sequence of human IL-6Rα-Fc

Sequence Range: 1 to 594

10 20 30 40 50 60

MVAVGCALLAALLAAPGAAL APRRCPAQEVARGVLTSLPG DSVTLTCPGVEPEDNATVHW

70 80 90 100 110 120

VLRKPAAGSHPSRWAGMGRR LLLRSVQLHDSGNYSCYRAG RPAGTVHLLVDVPPEEPQLS

130 140 150 160 170 180

CFRKSPLSNVVCEWGPRSTP SLTTKAVLLVRKFQNSPAED FQEPCQYSQESQKFSCQLAV

190 200 210 220 230 240

PEGDSSFYIVSMCVASSVGS KFSKTQTFQGCGILQPDPPA NITVTAVARNPRWLSVTWQD

250 260 270 280 290 300

PHSWNSSFYRLRFELRYRAE RSKTFTTWMVKDLQHHCVIH DAWSGLRHVVQLRAQEEFGQ

310 320 330 340 350 360

GEWSEWSPEAMGTPWTESRS PPAENEVSTPMQALTTNKDD DNILFRDSANATSLPVQDAG

370 † † 380 390 400 410 420

EPKSCDKTHTCPPCPAPELL GGPSVFLFPPKPKDTLMISR TPEVTCVVVDVSHEDPEVKF

430 440 450 460 470 480

NWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLN GKEYKCKVSNKALPAPIEKT

490 500 510 520 530 540

ISKAKGQPREPQVYTLPPSR DELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTP

550 560 570 580 590

PVLDSDGSFFLYSKLTVDKS RWQQGNVFSCSVMHEALHNH YTQKSLSLSPGK•

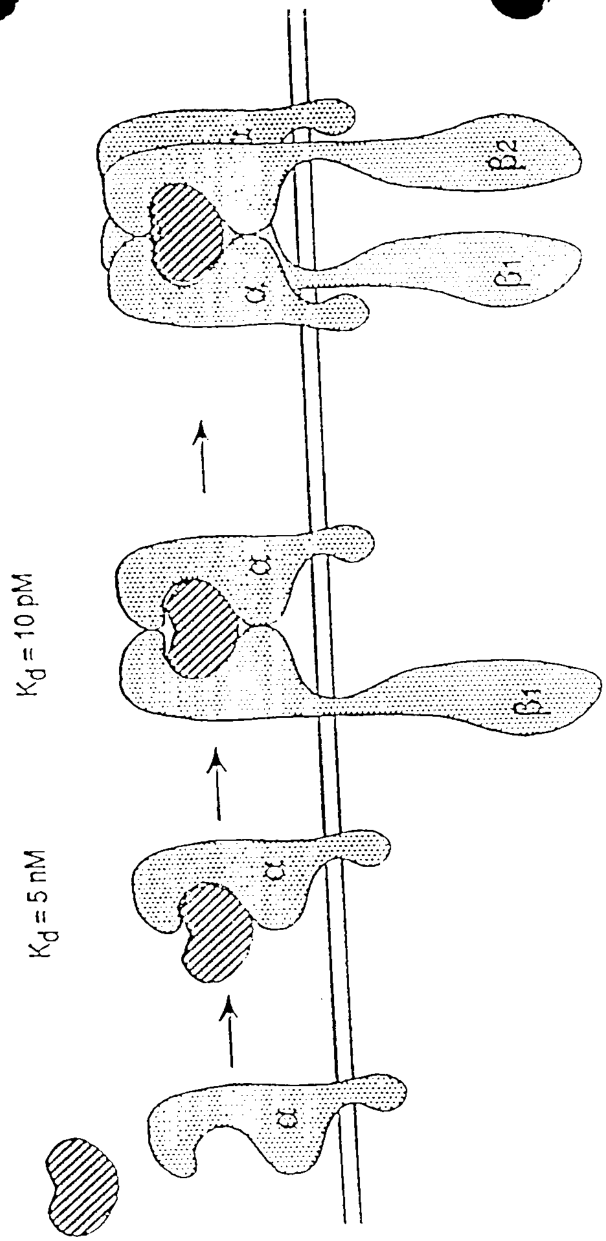


FIGURE 6

FIGURE 7
Heterodimeric Receptor-Based Ligand Trap

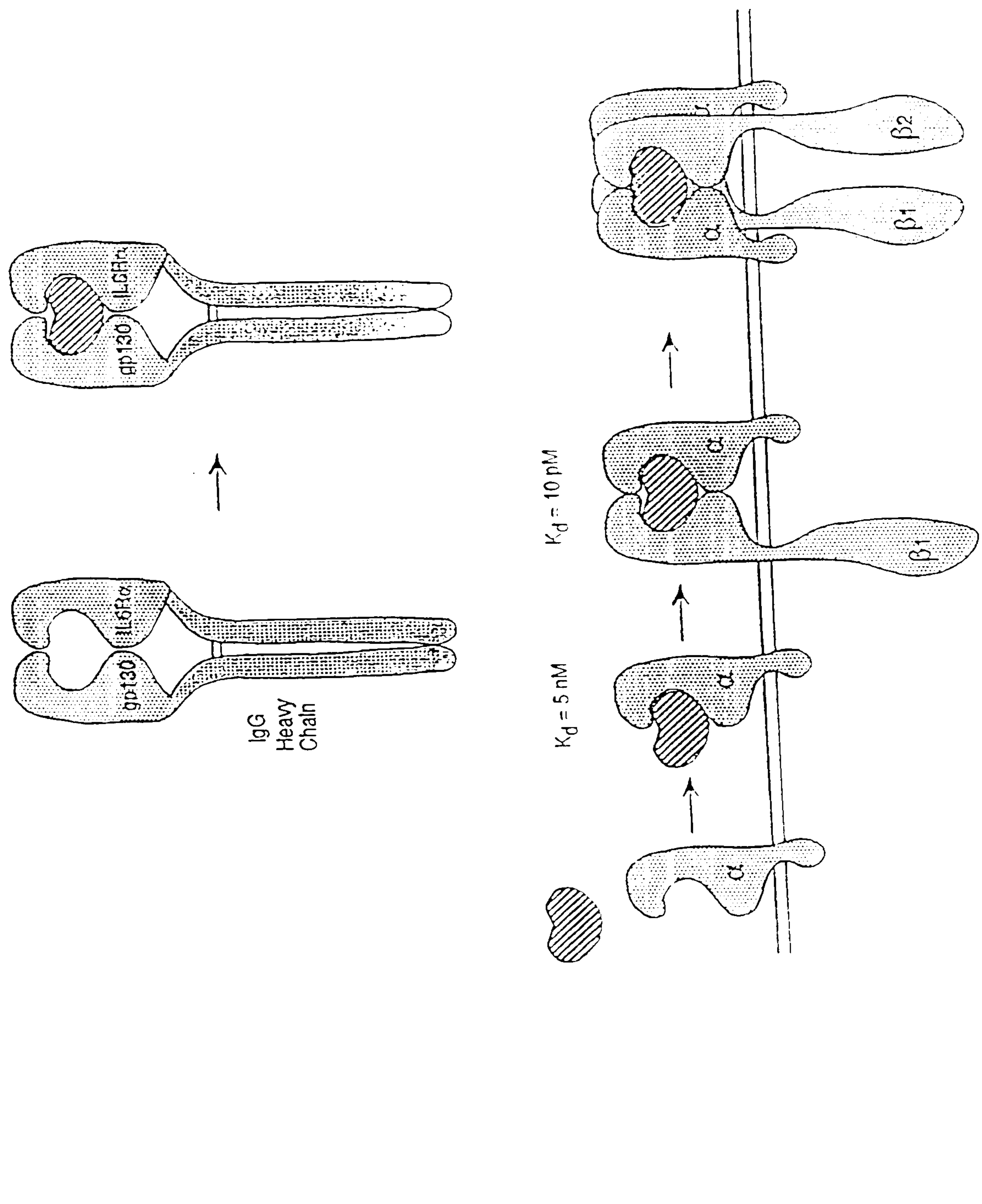


FIGURE 8

Immunoglobulin Heavy/Light Chain Receptor Fusions

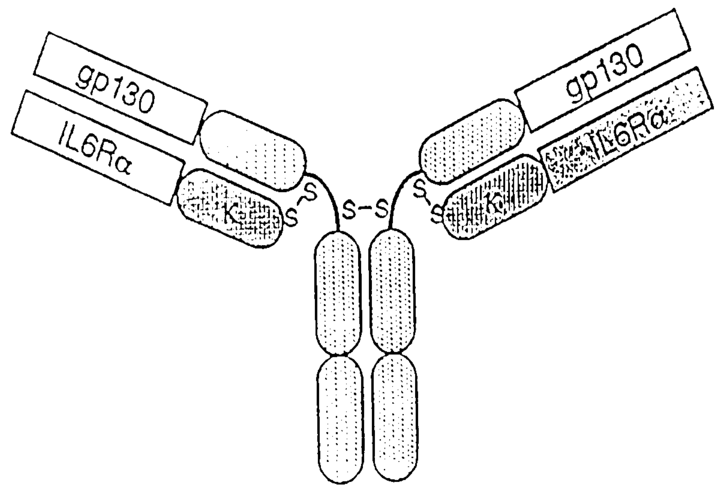


FIGURE 9

Amino acid sequence of gp130-Cγ1

```
Sequence Range: 1 to 952

        10         20         30         40         50         60
         *          *          *          *          *          *
MVTLQTWVVQALFIFLTTES TGELLDPCGYISPESPVVQL HSNFTAVCVLKEKCMDYFHV

        70         80         90        100        110        120
         *          *          *          *          *          *
NANYIVWKTNHFTIPKEQYT IINRTASSVTFTDIASLNIQ LTCNILTFGQLEQNVYGITI

       130        140        150        160        170        180
         *          *          *          *          *          *
ISGLPPEKPKNLSCIVNEGK KMRCEWDGGRETHLETNFTL KSEWATHKFADCKAKRDTPT

       190        200        210        220        230        240
         *          *          *          *          *          *
SCTVDYSTVYFVNIEVWVEA ENALGKVTSDHINFDPVYKV KPNPPHNLSVINSEELSSIL

       250        260        270        280        290        300
         *          *          *          *          *          *
KLTWTNPSIKSVIILKYNIQ YRTKDASTWSQIPPEDTAST RSSFTVQDLKPFTEYVFRIR

       310        320        330        340        350        360
         *          *          *          *          *          *
CMKEDGKGYWSDWSEEASGI TYEDRPSKAPSFWYKIDPSH TQGYRTVQLVWKTLPPFEAN

       370        380        390        400        410        420
         *          *          *          *          *          *
GKILDYEVTLTRWKSHLQNY TVNATKLTVNLTNDRYLATL TVRNLVGKSDAAVLTIPACD

       430        440        450        460        470        480
         *          *          *          *          *          *
FQATHPVMDLKAFPKDNMLW VEWTTPRESVKKYILEWCVL SDKAPCITDWQQEDGTVHRT

       490        500        510        520        530        540
         *          *          *          *          *          *
YLRGNLAESKCYLITVTPVY ADGPGSPESIKAYLKQAPPS KGPTVRTKKVGKNEAVLEWD

       550        560        570        580        590        600
         *          *          *          *          *          *
QLPVDVQNGFIRNYTIFYRT IIGNETAVNVDSSHTEYTLS SLTSDTLYMVRMAAYTDEGG

       610        620        630        640        650        660
         *          *          *          *          *          *
KDGPEFTFTTPKFAQGEIES GASTKGPSVFPLAPSSKSTS GGTAALGCLVKDYFPEPVTV

       670        680        690        700        710        720
         *          *          *          *          *          *
SWNSGALTSGVHTFPAVLQS SGLYSLSSVVTVPSSSLGTQ TYICNVNHKPSNTKVDKKVE

       730        740        750        760        770        780
         *          *          *          *          *          *
PKSCDKTHTCPPCPAPELLG GPSVFLFPPKPKDTLMISRT PEVTCVVVDVSHEDPEVKFN
```

FIGURE 9 continued

```
         790        800        810        820        830        840
           *          *          *          *          *          *
WYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTI

         850        860        870        880        890        900
           *          *          *          *          *          *
SKAKGQPREPQVYTLPPSRD ELTKNQVSLTCLVKGFYPSD IAVEWESNGQPENNYKTTPP

         910        920        930        940        950
           *          *          *          *          *
VLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHY TQKSLSLSPGK*
```

FIGURE 10

Amino acid sequence of gp130Δ3fibro

Sequence Range: 1 to 332

```
                 10                  20                  30                  40                  50                  60
                  *                   *                   *                   *                   *                   *
MVTLQTWVVQALFIFLTTES TGELLDPCGYISPESPVVQL HSNFTAVCVLKEKCMDYFHV

                 70                  80                  90                 100                 110                 120
                  *                   *                   *                   *                   *                   *
NANYIVWKTNHFTIPKEQYT IINRTASSVTFTDIASLNIQ LTCNILTFGQLEQNVYGITI

                130                 140                 150                 160                 170                 180
                  *                   *                   *                   *                   *                   *
ISGLPPEKPKNLSCIVNEGK KMRCEWDGGRETHLETNFTL KSEWATHKFADCKAKRDTPT

                190                 200                 210                 220                 230                 240
                  *                   *                   *                   *                   *                   *
SCTVDYSTVYFVNIEVWVEA ENALGKVTSDHINFDPVYKV KPNPPHNLSVINSEELSSIL

                250                 260                 270                 280                 290                 300
                  *                   *                   *                   *                   *                   *
KLTWTNPSIKSVIILKYNIQ YRTKDASTWSQIPPEDTAST RSSFTVQDLKPFTEYVFRIR

                310                 320                 330
                  *                   *                   *
CMKEDGKGYWSDWSEEASGI TYEDRPSKAPSG
```

FIGURE 11

Amino acid sequence of J-CH1

Sequence Range: 1 to 121

```
         10        20         30         40         50         60
          *         *          *          *          *          *
SGGQGTLVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCL VKDYFPEPVTVSWNSGALTS
         70        80         90        100        110        120
          *         *          *          *          *          *
GVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHK PSNTKVDKKVEPKSCDKTHT*
```

FIGURE 12

Amino acid sequence of Cγ4

Sequence Range: 1 to 330

```
                  10         20         30         40         50         60
                   *          *          *          *          *          *
SGASTKGPSVFPLAPCSRST SESTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQ
                  70         80         90        100        110        120
                   *          *          *          *          *          *
SSGLYSLSSVVTVPSSSLGT KTYTCNVDHKPSNTKVDKRV ESKYGPPCPSCPAPEFLGGP
                 130        140        150        160        170        180
                   *          *          *          *          *          *
SVFLFPPKPKDTLMISRTPE VTCVVVDVSQEDPEVQFNWY VDGVEVHNAKTKPREEQFNS
                 190        200        210        220        230        240
                   *          *          *          *          *          *
TYRVVSVLTVLHQDWLNGKE YKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEM
                 250        260        270        280        290        300
                   *          *          *          *          *          *
TKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVL DSDGSFFLYSRLTVDKSRWQ
                 310        320        330
                   *          *          *
EGNVFSCSVMHEALHNHYTQ KSLSLSLGK*
```

FIGURE 13

Amino acid sequence of κ-domain

Sequence Range: 1 to 108

```
         10         20         30         40         50         60
          *          *          *          *          *          *
SGTVAAPSVFIFPPSDEQLK SGTASVVCLLNNFYPREAKV QWKVDNALQSGNSQESVTEQ

         70         80         90        100
          *          *          *          *
DSKDSTYSLSSTLTLSKADY EKHKVYACEVTHQGLSSPVT KSFNRGEC*
```

FIGURE 14

Amino acid sequence of λ-domain:

```
Sequence Range: 1 to 107

        10         20         30         40         50         60
         *          *          *          *          *          *
SGPKAAPSVTLFPPSSEELQ ANKATLVCLISDFYPGAVTV AWKADSSPVKAGVETTTPSK

        70         80         90        100
         *          *          *          *
QSNNKYAASSYLSLTPEQWK SHRSYSCQVTHEGSTVEKTV APTECS*
```

FIGURE 15

Amino acid sequence of the soluble IL-6Rα domain

Sequence Range: 1 to 360

```
         10         20         30         40         50         60
          *          *          *          *          *          *
MVAVGCALLAALLAAPGAAL APRRCPAQEVARGVLTSLPG DSVTLTCPGVEPEDNATVHW

         70         80         90        100        110        120
          *          *          *          *          *          *
VLRKPAAGSHPSRWAGMGRR LLLRSVQLHDSGNYSCYRAG RPAGTVHLLVDVPPEEPQLS

        130        140        150        160        170        180
          *          *          *          *          *          *
CFRKSPLSNVVCEWGPRSTP SLTTKAVLLVRKFQNSPAED FQEPCQYSQESQKFSCQLAV

        190        200        210        220        230        240
          *          *          *          *          *          *
PEGDSSFYIVSMCVASSVGS KFSKTQTFQGCGILQPDPPA NITVTAVARNPRWLSVTWQD

        250        260        270        280        290        300
          *          *          *          *          *          *
PHSWNSSFYRLRFELRYRAE RSKTFTTWMVKDLQHHCVIH DAWSGLRHVVQLRAQEEFGQ

        310        320        330        340        350        360
          *          *          *          *          *          *
GEWSEWSPEAMGTPWTESRS PPAENEVSTPMQALTTNKDD DNILFRDSANATSLPVQDAG
```

FIGURE 16

Amino acid sequence of the soluble IL-6Rα313 domain

Sequence Range: 1 to 315

```
                10         20         30         40         50         60
                 *          *          *          *          *          *
MVAVGCALLAALLAAPGAAL APRRCPAQEVARGVLTSLPG DSVTLTCPGVEPEDNATVHW

                70         80         90        100        110        120
                 *          *          *          *          *          *
VLRKPAAGSHPSRWAGMGRR LLLRSVQLHDSGNYSCYRAG RPAGTVHLLVDVPPEEPQLS

               130        140        150        160        170        180
                 *          *          *          *          *          *
CFRKSPLSNVVCEWGPRSTP SLTTKAVLLVRKFQNSPAED FQEPCQYSQESQKFSCQLAV

               190        200        210        220        230        240
                 *          *          *          *          *          *
PEGDSSFYIVSMCVASSVGS KFSKTQTFQGCGILQPDPPA NITVTAVARNPRWLSVTWQD

               250        260        270        280        290        300
                 *          *          *          *          *          *
PHSWNSSFYRLRFELRYRAE RSKTFTTWMVKDLQHHCVIH DAWSGLRHVVQLRAQEEFGQ

               310
                 *
GEWSEWSPEAMGTTG
```

FIGURE 17

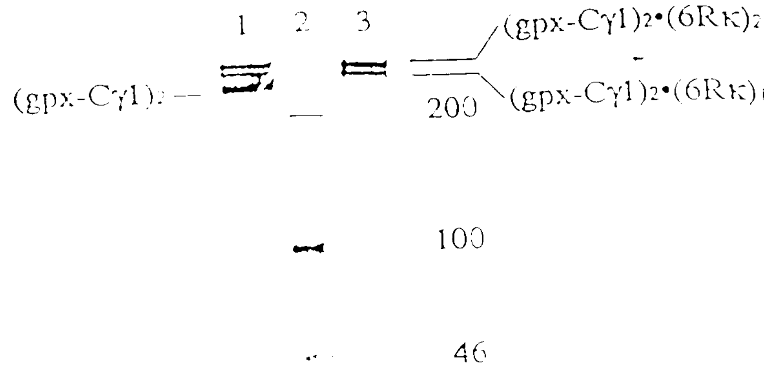


FIGURE 18

IL-6 Dissociates Slowly from the Ligand Trap

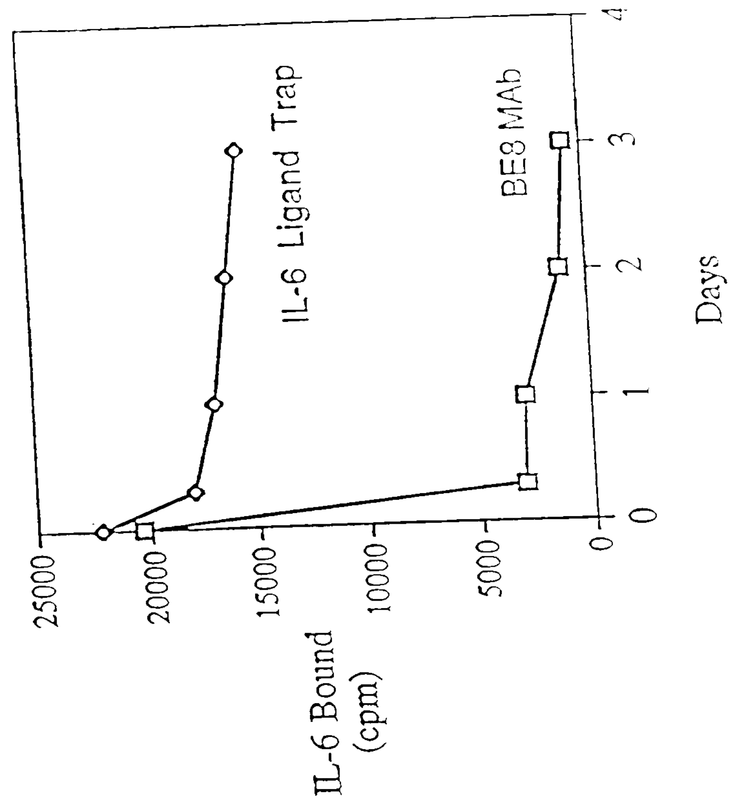


FIGURE 19

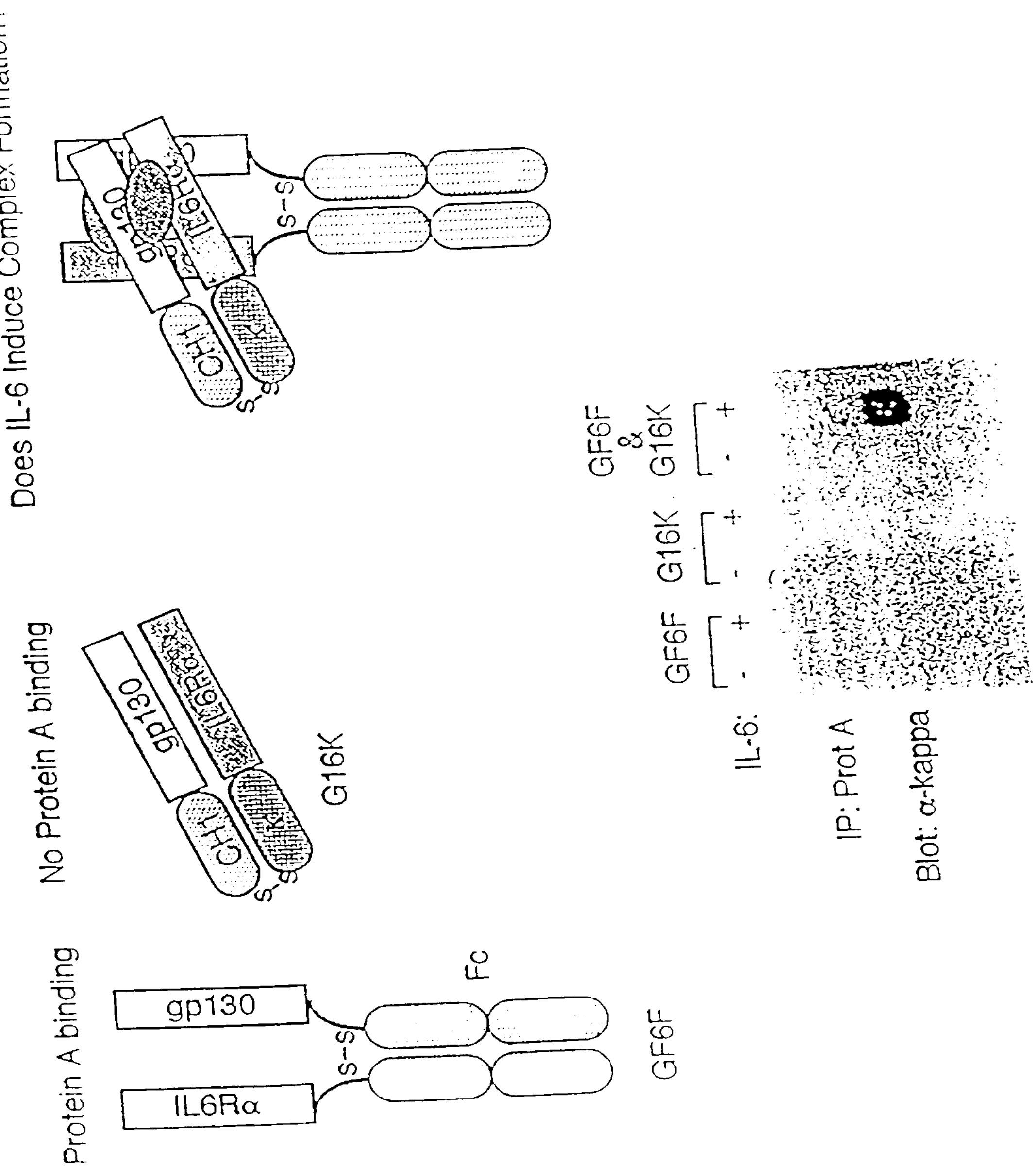

Protein A binding
gp130
IL6Rα
s–s
Fc
GF6F
No Protein A binding
gp130
IL6Rα
CH1
K
s–s
G16K
Does IL-6 Induce Complex Formation?
gp130
IL6Rα
CH1
K
s–s
IL-6:
GF6F
- +
G16K
- +
GF6F & G16K
- +
IP: Prot A
Blot: α-kappa

FIGURE 20

XG-1 cell proliferation assay

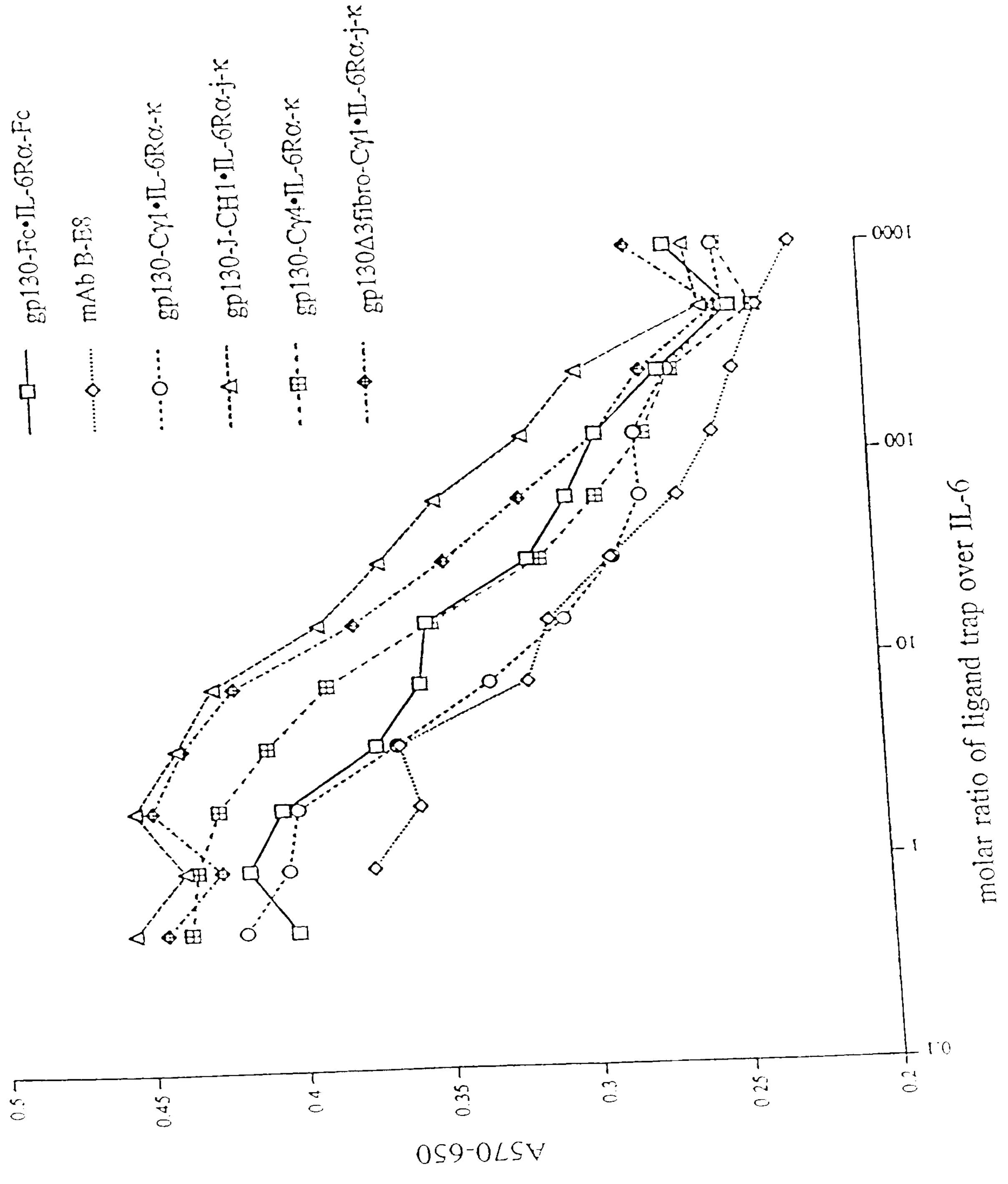


Figure 21A

```
                  10                  20                  30                  40
ATG GTG AAG CCA TCA TTA CCA TTC ACA TCC CTT TTA TTC CTG CAG CTG
Met Val Lys Pro Ser Leu Pro Phe Thr Ser Leu Leu Phe Leu Gln Leu>

50                  60                  70                  80                  90
CCC CTG CTG GGA GTG GGG CTG AAC ACG ACA ATT CTG ACG CCC AAT GGG
Pro Leu Leu Gly Val Gly Leu Asn Thr Thr Ile Leu Thr Pro Asn Gly>

  100                 110                 120                 130                 140
AAT GAA GAC ACC ACA GCT GAT TTC TTC CTG ACC ACT ATG CCC ACT GAC
Asn Glu Asp Thr Thr Ala Asp Phe Phe Leu Thr Thr Met Pro Thr Asp>

     150                 160                 170                 180                 190
TCC CTC AGT GTT TCC ACT CTG CCC CTC CCA GAG GTT CAG TGT TTT GTG
Ser Leu Ser Val Ser Thr Leu Pro Leu Pro Glu Val Gln Cys Phe Val>

        200                 210                 220                 230                 240
TTC AAT GTC GAG TAC ATG AAT TGC ACT TGG AAC AGC AGC TCT GAG CCC
Phe Asn Val Glu Tyr Met Asn Cys Thr Trp Asn Ser Ser Ser Glu Pro>

           250                 260                 270                 280
CAG CCT ACC AAC CTC ACT CTG CAT TAT TGG TAC AAG AAC TCG GAT AAT
Gln Pro Thr Asn Leu Thr Leu His Tyr Trp Tyr Lys Asn Ser Asp Asn>

290                 300                 310                 320                 330
GAT AAA GTC CAG AAG TGC AGC CAC TAT CTA TTC TCT GAA GAA ATC ACT
Asp Lys Val Gln Lys Cys Ser His Tyr Leu Phe Ser Glu Glu Ile Thr>

  340                 350                 360                 370                 380
TCT GGC TGT CAG TTG CAA AAA AAG GAG ATC CAC CTC TAC CAA ACA TTT
Ser Gly Cys Gln Leu Gln Lys Lys Glu Ile His Leu Tyr Gln Thr Phe>

     390                 400                 410                 420                 430
GTT GTT CAG CTC CAG GAC CCA CGG GAA CCC AGG AGA CAG GCC ACA CAG
Val Val Gln Leu Gln Asp Pro Arg Glu Pro Arg Arg Gln Ala Thr Gln>

        440                 450                 460                 470                 480
ATG CTA AAA CTG CAG AAT CTG GTG ATC CCC TGG GCT CCA GAG AAC CTA
Met Leu Lys Leu Gln Asn Leu Val Ile Pro Trp Ala Pro Glu Asn Leu>

           490                 500                 510                 520
ACA CTT CAC AAA CTG AGT GAA TCT CAG CTA GAA CTG AAC TGG AAC AAC
Thr Leu His Lys Leu Ser Glu Ser Gln Leu Glu Leu Asn Trp Asn Asn>

530                 540                 550                 560                 570
AGA TTC TTG AAC CAC TGT TTG GAG CAC TTG GTG CAG TAC CGG ACT GAC
Arg Phe Leu Asn His Cys Leu Glu His Leu Val Gln Tyr Arg Thr Asp>
```

```
580         590         600         610         620
TGG GAC CAC AGC TGG ACT GAA CAA TCA GTG GAT TAT AGA CAT AAG TTC
Trp Asp His Ser Trp Thr Glu Gln Ser Val Asp Tyr Arg His Lys Phe>

    630         640         650         660         670
TCC TTG CCT AGT GTG GAT GGG CAG AAA CGC TAC ACG TTT CGT GTT CGG
Ser Leu Pro Ser Val Asp Gly Gln Lys Arg Tyr Thr Phe Arg Val Arg>

        680         690         700         710         720
AGC CGC TTT AAC CCA CTC TGT GGA AGT GCT CAG CAT TGG AGT GAA TGG
Ser Arg Phe Asn Pro Leu Cys Gly Ser Ala Gln His Trp Ser Glu Trp>

            730         740         750         760
AGC CAC CCA ATC CAC TGG GGG AGC AAT ACT TCA AAA GAG AAC GCG TCG
Ser His Pro Ile His Trp Gly Ser Asn Thr Ser Lys Glu Asn Ala Ser>

770         780         790         800         810
TCT GGG AAC ATG AAG GTC CTG CAG GAG CCC ACC TGC GTC TCC GAC TAC
Ser Gly Asn Met Lys Val Leu Gln Glu Pro Thr Cys Val Ser Asp Tyr>

    820         830         840         850         860
ATG AGC ATC TCT ACT TGC GAG TGG AAG ATG AAT GGT CCC ACC AAT TGC
Met Ser Ile Ser Thr Cys Glu Trp Lys Met Asn Gly Pro Thr Asn Cys>

        870         880         890         900         910
AGC ACC GAG CTC CGC CTG TTG TAC CAG CTG GTT TTT CTG CTC TCC GAA
Ser Thr Glu Leu Arg Leu Leu Tyr Gln Leu Val Phe Leu Leu Ser Glu>

            920         930         940         950         960
GCC CAC ACG TGT ATC CCT GAG AAC AAC GGA GGC GCG GGG TGC GTG TGC
Ala His Thr Cys Ile Pro Glu Asn Asn Gly Gly Ala Gly Cys Val Cys>

                970         980         990         1000
CAC CTG CTC ATG GAT GAC GTG GTC AGT GCG GAT AAC TAT ACA CTG GAC
His Leu Leu Met Asp Asp Val Val Ser Ala Asp Asn Tyr Thr Leu Asp>

1010        1020        1030        1040        1050
CTG TGG GCT GGG CAG CAG CTG CTG TGG AAG GGC TCC TTC AAG CCC AGC
Leu Trp Ala Gly Gln Gln Leu Leu Trp Lys Gly Ser Phe Lys Pro Ser>

    1060        1070        1080        1090        1100
GAG CAT GTG AAA CCC AGG GCC CCA GGA AAC CTG ACA GTT CAC ACC AAT
Glu His Val Lys Pro Arg Ala Pro Gly Asn Leu Thr Val His Thr Asn>

        1110        1120        1130        1140        1150
GTC TCC GAC ACT CTG CTG CTG ACC TGG AGC AAC CCG TAT CCC CCT GAC
Val Ser Asp Thr Leu Leu Leu Thr Trp Ser Asn Pro Tyr Pro Pro Asp>

            1160        1170        1180        1190        1200
```

```
AAT TAC CTG TAT AAT CAT CTC ACC TAT GCA GTC AAC ATT TGG AGT GAA
Asn Tyr Leu Tyr Asn His Leu Thr Tyr Ala Val Asn Ile Trp Ser Glu>

        1210        1220        1230        1240
AAC GAC CCG GCA GAT TTC AGA ATC TAT AAT GTG ACC TAC CTA GAA CCC
Asn Asp Pro Ala Asp Phe Arg Ile Tyr Asn Val Thr Tyr Leu Glu Pro>

1250        1260        1270        1280        1290
TCC CTC CGC ATC GCA GCC AGC ACC CTG AAG TCT GGG ATT TCC TAC AGG
Ser Leu Arg Ile Ala Ala Ser Thr Leu Lys Ser Gly Ile Ser Tyr Arg>

 1300        1310        1320        1330        1340
GCA CGG GTG AGG GCC TGG GCT CAG TGC TAT AAC ACC ACC TGG AGT GAG
Ala Arg Val Arg Ala Trp Ala Gln Cys Tyr Asn Thr Thr Trp Ser Glu>

   1350        1360        1370        1380        1390
TGG AGC CCC AGC ACC AAG TGG CAC AAC TCC TAC AGG GAG CCC TTC GAG
Trp Ser Pro Ser Thr Lys Trp His Asn Ser Tyr Arg Glu Pro Phe Glu>

     1400        1410        1420        1430        1440
CAG TCC GGA GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA
Gln Ser Gly Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu>

        1450        1460        1470        1480
CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC
Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp>

1490        1500        1510        1520        1530
ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC
Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp>

 1540        1550        1560        1570        1580
GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC
Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly>

   1590        1600        1610        1620        1630
GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC
Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn>

      1640        1650        1660        1670        1680
AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG
Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp>

         1690        1700        1710        1720
CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA
Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro>

1730        1740        1750        1760        1770
GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA
Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu>
```

Figure 21D

```
1780         1790         1800         1810         1820
CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAG GAG ATG ACC AAG AAC
Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn>

    1830         1840         1850         1860         1870
CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC
Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile>

        1880         1890         1900         1910         1920
GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC
Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr>

            1930         1940         1950         1960
ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAT AGC AAG
Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys>

1970         1980         1990         2000         2010
CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC
Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys>

    2020         2030         2040         2050         2060
TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC
Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu>

        2070         2080
TCC CTG TCT CCG GGT AAA TGA
Ser Leu Ser Pro Gly Lys ***>
```

Figure 22A

```
            10                  20                  30                  40
ATG GTG AAG CCA TCA TTA CCA TTC ACA TCC CTC TTA TTC CTG CAG CTG
Met Val Lys Pro Ser Leu Pro Phe Thr Ser Leu Leu Phe Leu Gln Leu>

50                  60                  70                  80                  90
CCC CTG CTG GGA GTG GGG CTG AAC ACG ACA ATT CTG ACG CCC AAT GGG
Pro Leu Leu Gly Val Gly Leu Asn Thr Thr Ile Leu Thr Pro Asn Gly>

    100                 110                 120                 130                 140
AAT GAA GAC ACC ACA GCT GAT TTC TTC CTG ACC ACT ATG CCC ACT GAC
Asn Glu Asp Thr Thr Ala Asp Phe Phe Leu Thr Thr Met Pro Thr Asp>

    150                 160                 170                 180                 190
TCC CTC AGT GTT TCC ACT CTG CCC CTC CCA GAG GTT CAG TGT TTT GTG
Ser Leu Ser Val Ser Thr Leu Pro Leu Pro Glu Val Gln Cys Phe Val>

        200                 210                 220                 230                 240
TTC AAT GTC GAG TAC ATG AAT TGC ACT TGG AAC AGC AGC TCT GAG CCC
Phe Asn Val Glu Tyr Met Asn Cys Thr Trp Asn Ser Ser Ser Glu Pro>

            250                 260                 270                 280
CAG CCT ACC AAC CTC ACT CTG CAT TAT TGG TAC AAG AAC TCG GAT AAT
Gln Pro Thr Asn Leu Thr Leu His Tyr Trp Tyr Lys Asn Ser Asp Asn>

290                 300                 310                 320                 330
GAT AAA GTC CAG AAG TGC AGC CAC TAT CTA TTC TCT GAA GAA ATC ACT
Asp Lys Val Gln Lys Cys Ser His Tyr Leu Phe Ser Glu Glu Ile Thr>

    340                 350                 360                 370                 380
TCT GGC TGT CAG TTG CAA AAA AAG GAG ATC CAC CTC TAC CAA ACA TTT
Ser Gly Cys Gln Leu Gln Lys Lys Glu Ile His Leu Tyr Gln Thr Phe>

    390                 400                 410                 420                 430
GTT GTT CAG CTC CAG GAC CCA CGG GAA CCC AGG AGA CAG GCC ACA CAG
Val Val Gln Leu Gln Asp Pro Arg Glu Pro Arg Arg Gln Ala Thr Gln>

        440                 450                 460                 470                 480
ATG CTA AAA CTG CAG AAT CTG GTG ATC CCC TGG GCT CCA GAG AAC CTA
Met Leu Lys Leu Gln Asn Leu Val Ile Pro Trp Ala Pro Glu Asn Leu>

            490                 500                 510                 520
ACA CTT CAC AAA CTG AGT GAA TCC CAG CTA GAA CTT AAC TGG AAC AAC
Thr Leu His Lys Leu Ser Glu Ser Gln Leu Glu Leu Asn Trp Asn Asn>

530                 540                 550                 560                 570
AGA TTC TTG AAC CAC TGT TTG GAG CAC TTG GTG CAG TAC CGG ACT GAC
Arg Phe Leu Asn His Cys Leu Glu His Leu Val Gln Tyr Arg Thr Asp>
```

Figure 22B

580 590 600 610 620

TGG GAC CAC AGC TGG ACT GAA CAA TCA GTG GAT TAT AGA CAT AAG TTC
Trp Asp His Ser Trp Thr Glu Gln Ser Val Asp Tyr Arg His Lys Phe>

630 640 650 660 670

TCC TTG [illegible] AGT GTG GAT GGG CAG AAA CGC TAT ACG TTT CGT GTT CGG
Ser Leu Pro Ser Val Asp Gly Gln Lys Arg Tyr Thr Phe Arg Val Arg>

680 690 700 710 720

AGC CGC TTT AAC CCA CTC TGT GGA AGT GCT CAG CAT TGG AGT GAA TGG
Ser Arg Phe Asn Pro Leu Cys Gly Ser Ala Gln His Trp Ser Glu Trp>

730 740 750 760

AGC CAC CCA ATC CAC TGG GGG AGC AAT ACT TCA AAA GAG AAC GGG AAC
Ser His Pro Ile His Trp Gly Ser Asn Thr Ser Lys Glu Asn Gly Asn>

770 780 790 800 810

ATG AAG GTC CTG CAG GAG CCC ACC TGC GTC TCC GAC TAC ATG AGC ATC
Met Lys Val Leu Gln Glu Pro Thr Cys Val Ser Asp Tyr Met Ser Ile>

820 830 840 850 860

TCT ACT TGC GAG TGG AAG ATG AAT GGT CCC ACC AAT TGC AGC ACC GAG
Ser Thr Cys Glu Trp Lys Met Asn Gly Pro Thr Asn Cys Ser Thr Glu>

870 880 890 900 910

CTC CGC CTG TTG TAC CAG CTG GTT TTT CTG CTC TCC GAA GCC CAC ACG
Leu Arg Leu Leu Tyr Gln Leu Val Phe Leu Leu Ser Glu Ala His Thr>

920 930 940 950 960

TGT ATC CCT GAG AAC AAC GGA GGC GCG GGG TGC GTG TGC CAC CTG CTC
Cys Ile Pro Glu Asn Asn Gly Gly Ala Gly Cys Val Cys His Leu Leu>

970 980 990 1000

ATG GAT GAC GTG GTC AGT GCG GAT AAC TAT ACA CTG GAC CTG TGG GCT
Met Asp Asp Val Val Ser Ala Asp Asn Tyr Thr Leu Asp Leu Trp Ala>

1010 1020 1030 1040 1050

GGG CAG CAG CTG CTG TGG AAG GGC TCC TTC AAG CCC AGC GAG CAT GTG
Gly Gln Gln Leu Leu Trp Lys Gly Ser Phe Lys Pro Ser Glu His Val>

1060 1070 1080 1090 1100

AAA CCC AGG GCC CCA GGA AAC CTG ACA GTT CAC ACC AAT GTC TCC GAC
Lys Pro Arg Ala Pro Gly Asn Leu Thr Val His Thr Asn Val Ser Asp>

1110 1120 1130 1140 1150

ACT CTG CTG CTG ACC TGG AGC AAC CCG TAT CCC CCT GAC AAT TAC CTG
Thr Leu Leu Leu Thr Trp Ser Asn Pro Tyr Pro Pro Asp Asn Tyr Leu>

1160 1170 1180 1190 1200

Figure 22C

TAT AAT CAT CTC ACC TAT GCA GTC AAC ATT TGG AGT GAA AAC GAC CCG
Tyr Asn His Leu Thr Tyr Ala Val Asn Ile Trp Ser Glu Asn Asp Pro>

GCA GAT TTC AGA ATC TAT AAC GTG ACC TAC CTA GAA CCC TCC CTC CGC
Ala Asp Phe Arg Ile Tyr Asn Val Thr Tyr Leu Glu Pro Ser Leu Arg>

ATC GCA GCC AGC ACC CTG AAG TCT GGG ATT TCC TAC AGG GCA CGG GTG
Ile Ala Ala Ser Thr Leu Lys Ser Gly Ile Ser Tyr Arg Ala Arg Val>

AGG GCC TGG GCT CAG AGC TAT AAC ACC ACC TGG AGT GAG TGG AGC CCC
Arg Ala Trp Ala Gln Ser Tyr Asn Thr Thr Trp Ser Glu Trp Ser Pro>

AGC ACC AAG TGG CAC AAC TCC TAC AGG GAG CCC TTC GAG CAG TCC GGA
Ser Thr Lys Trp His Asn Ser Tyr Arg Glu Pro Phe Glu Gln Ser Gly>

GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly>

GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG
Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met>

ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC
Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His>

GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG
Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val>

CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC
His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr>

CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC
Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly>

AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC
Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile>

GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG
Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val>

Figure 22D

```
1780      1790      1800      1810      1820
TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC
Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser>

   1830      1840      1850      1860      1870
CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG
Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu>

   1880      1890      1900      1910      1920
TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro>

      1930      1940      1950      1960
GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAT AGC AAG CTC ACC GTG
Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val>

1970      1980      1990      2000      2010
GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG
Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met>

 2020      2030      2040      2050      2060
CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT
His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser>

   2070
CCG GGT AAA TGA
Pro Gly Lys ***>
```

Figure 23A

```
         10          20          30          40
ATG GTG AAG CCA TCA TTA CCA TTC ACA TCT CTC TTA TTC CTG CAG CTG
Met Val Lys Pro Ser Leu Pro Phe Thr Ser Leu Leu Phe Leu Gln Leu>

50          60          70          80          90
CCC CTG CTG GGA GTG GGG CTG AAC ACG ACA ATT CTG ACG CCC AAT GGG
Pro Leu Leu Gly Val Gly Leu Asn Thr Thr Ile Leu Thr Pro Asn Gly>

 100         110         120         130         140
AAT GAA GAC ACC ACA GCT GAT TTC TTC CTG ACC ACT ATG CCC ACT GAC
Asn Glu Asp Thr Thr Ala Asp Phe Phe Leu Thr Thr Met Pro Thr Asp>

    150         160         170         180         190
TCC CTC AGT GTT TCC ACT CTG CCC CTC CCA GAG GTT CAG TGT TTT GTG
Ser Leu Ser Val Ser Thr Leu Pro Leu Pro Glu Val Gln Cys Phe Val>

       200         210         220         230         240
TTC AAT GTC GAG TAC ATG AAT TGC ACT TGG AAC AGC AGC TCT GAG CCC
Phe Asn Val Glu Tyr Met Asn Cys Thr Trp Asn Ser Ser Ser Glu Pro>

          250         260         270         280
CAG CCT ACC AAC CTC ACT CTG CAT TAT TGG TAC AAG AAC TCG GAT AAT
Gln Pro Thr Asn Leu Thr Leu His Tyr Trp Tyr Lys Asn Ser Asp Asn>

290         300         310         320         330
GAT AAA GTC CAG AAG TGC AGC CAC TAT CTA TTC TCT GAA GAA ATC ACT
Asp Lys Val Gln Lys Cys Ser His Tyr Leu Phe Ser Glu Glu Ile Thr>

 340         350         360         370         380
TCT GGC TGT CAG TTG CAA AAA AAG GAG ATC CAC CTC TAC CAA ACA TTT
Ser Gly Cys Gln Leu Gln Lys Lys Glu Ile His Leu Tyr Gln Thr Phe>

    390         400         410         420         430
GTT GTT CAG CTC CAG GAC CCA CGG GAA CCC AGG AGA CAG GCC ACA CAG
Val Val Gln Leu Gln Asp Pro Arg Glu Pro Arg Arg Gln Ala Thr Gln>

       440         450         460         470         480
ATG CTA AAA CTG CAG AAT CTG GTG ATC CCC TGG GCT CCA GAG AAC CTA
Met Leu Lys Leu Gln Asn Leu Val Ile Pro Trp Ala Pro Glu Asn Leu>

          490         500         510         520
ACA CTT CAC AAA CTG AGT GAA TCC CAG CTA GAA CTG AAC TGG AAC AAC
Thr Leu His Lys Leu Ser Glu Ser Gln Leu Glu Leu Asn Trp Asn Asn>

530         540         550         560         570
AGA TTC TTG AAC CAC TGT TTG GAG CAC TTG GTG CAG TAC CGG ACT GAC
Arg Phe Leu Asn His Cys Leu Glu His Leu Val Gln Tyr Arg Thr Asp>
```

Figure 23B

580 590 600 610 620

TGG GAC CAC AGC TGG ACT GAA CAA TCA GTG GAT TAT AGA CAT AAG TTC
Trp Asp His Ser Trp Thr Glu Gln Ser Val Asp Tyr Arg His Lys Phe>

630 640 650 660 670

TCC TTG CCT AGT GTG GAT GGG CAG AAA CGT TAT ACG TTT CGT GTT CGG
Ser Leu Pro Ser Val Asp Gly Gln Lys Arg Tyr Thr Phe Arg Val Arg>

680 690 700 710 720

AGC CGC TTT AAT CCA CTC TGT GGA AGT GCT CAG CAT TGG AGT GAA TGG
Ser Arg Phe Asn Pro Leu Cys Gly Ser Ala Gln His Trp Ser Glu Trp>

730 740 750 760

AGC CAC CCA ATC CAC TGG GGG AGC AAT ACT TCA AAA GAG AAC GCG TCG
Ser His Pro Ile His Trp Gly Ser Asn Thr Ser Lys Glu Asn Ala Ser>

770 780 790 800 810

TCT GGG AAC ATG AAG GTC CTG CAG GAG CCC ACC TGC GTC TCC GAC TAC
Ser Gly Asn Met Lys Val Leu Gln Glu Pro Thr Cys Val Ser Asp Tyr>

820 830 840 850 860

ATG AGC ATC TCT ACT TGC GAG TGG AAG ATG AAT GGT CCC ACC AAT TGC
Met Ser Ile Ser Thr Cys Glu Trp Lys Met Asn Gly Pro Thr Asn Cys>

870 880 890 900 910

AGC ACC GAG CTC CGC CTG TTG TAC CAG CTG GTT TTT CTG CTC TCC GAA
Ser Thr Glu Leu Arg Leu Leu Tyr Gln Leu Val Phe Leu Leu Ser Glu>

920 930 940 950 960

GCC CAC ACG TGT ATC CCT GAG AAC AAC GGA GGC GCG GGG TGC GTG TGC
Ala His Thr Cys Ile Pro Glu Asn Asn Gly Gly Ala Gly Cys Val Cys>

970 980 990 1000

CAC CTG CTC ATG GAT GAC GTG GTC AGT GCG GAT AAC TAT ACA CTG GAC
His Leu Leu Met Asp Asp Val Val Ser Ala Asp Asn Tyr Thr Leu Asp>

1010 1020 1030 1040 1050

CTG TGG GCT GGG CAG CAG CTG CTG TGG AAG GGC TCC TTC AAG CCC AGC
Leu Trp Ala Gly Gln Gln Leu Leu Trp Lys Gly Ser Phe Lys Pro Ser>

1060 1070 1080 1090 1100

GAG CAT GTG AAA CCC AGG GCC CCA GGA AAC CTG ACA GTT CAC ACC AAT
Glu His Val Lys Pro Arg Ala Pro Gly Asn Leu Thr Val His Thr Asn>

1110 1120 1130 1140 1150

GTC TCC GAC ACT CTG CTG CTG ACC TGG AGC AAC CCG TAT CCC CCT GAC
Val Ser Asp Thr Leu Leu Leu Thr Trp Ser Asn Pro Tyr Pro Pro Asp>

1160 1170 1180 1190 1200

Figure 23C

AAT TAC CTG TAT AAT CAT CTC ACC TAT GCA GTC AAC ATT TGG AGT GAA
Asn Tyr Leu Tyr Asn His Leu Thr Tyr Ala Val Asn Ile Trp Ser Glu>

1210 1220 1230 1240

AAC GAC CCG GCA GAT TTC AGA ATC TAT AAC GTG ACC TAC CTA GAA CCC
Asn Asp Pro Ala Asp Phe Arg Ile Tyr Asn Val Thr Tyr Leu Glu Pro>

1250 1260 1270 1280 1290

TCC CTC CGC ATC GCA GCC AGC ACC CTG AAG TCT GGG ATT TCC TAC AGG
Ser Leu Arg Ile Ala Ala Ser Thr Leu Lys Ser Gly Ile Ser Tyr Arg>

1300 1310 1320 1330 1340

GCA CGG GTG AGG GCC TGG GCT CAG AGC TAT AAC ACC ACC TGG AGT GAG
Ala Arg Val Arg Ala Trp Ala Gln Ser Tyr Asn Thr Thr Trp Ser Glu>

1350 1360 1370 1380 1390

TGG AGC CCC AGC ACC AAG TGG CAC AAC TCC TAC AGG GAG CCC TTC GAG
Trp Ser Pro Ser Thr Lys Trp His Asn Ser Tyr Arg Glu Pro Phe Glu>

1400 1410 1420 1430 1440

CAG TCC GGA GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA
Gln Ser Gly Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu>

1450 1460 1470 1480

CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC
Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp>

1490 1500 1510 1520 1530

ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC
Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp>

1540 1550 1560 1570 1580

GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC
Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly>

1590 1600 1610 1620 1630

GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC
Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn>

1640 1650 1660 1670 1680

AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG
Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp>

1690 1700 1710 1720

CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA
Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro>

1730 1740 1750 1760 1770

GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA
Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu>

Figure 23D

```
1780       1790       1800       1810       1820
CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC
Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn>

1830       1840       1850       1860       1870
CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC
Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile>

1880       1890       1900       1910       1920
GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC
Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr>

1930       1940       1950       1960
ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAT AGC AAG
Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys>

1970       1980       1990       2000       2010
CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC
Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys>

2020       2030       2040       2050       2060
TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC
Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu>

2070       2080
TCC CTG TCT CCG GGT AAA TGA
Ser Leu Ser Pro Gly Lys ***>
```

Figure 24A

10 20 30 40
ATG GTG GCC GTC GGC TGC GCG CTG CTG GCT GCT CTG CTG GCC GCG CCG
Met Val Ala Val Gly Cys Ala Leu Leu Ala Ala Leu Leu Ala Ala Pro>

50 60 70 80 90
GGA GCG GCG CTG GCC CCA AGG CGC TGC CCT GCG CAG GAG GTG GCA AGA
Gly Ala Ala Leu Ala Pro Arg Arg Cys Pro Ala Gln Glu Val Ala Arg>

100 110 120 130 140
GGC GTG CTG ACC AGT CTG CCA GGA GAC AGC GTG ACT CTG ACC TGC CCG
Gly Val Leu Thr Ser Leu Pro Gly Asp Ser Val Thr Leu Thr Cys Pro>

150 160 170 180 190
GGG GTA GAG CCG GAA GAC AAT GCC ACT GTT CAC TGG GTG CTC AGG AAG
Gly Val Glu Pro Glu Asp Asn Ala Thr Val His Trp Val Leu Arg Lys>

200 210 220 230 240
CCG GCT GCA GGC TCC CAC CCC AGC AGA TGG GCT GGC ATG GGA AGG AGG
Pro Ala Ala Gly Ser His Pro Ser Arg Trp Ala Gly Met Gly Arg Arg>

250 260 270 280
CTG CTG CTG AGG TCG GTG CAG CTC CAC GAC TCT GGA AAC TAT TCA TGC
Leu Leu Leu Arg Ser Val Gln Leu His Asp Ser Gly Asn Tyr Ser Cys>

290 300 310 320 330
TAC CGG GCC GGC CGC CCA GCT GGG ACT GTG CAC TTG CTG GTG GAT GTT
Tyr Arg Ala Gly Arg Pro Ala Gly Thr Val His Leu Leu Val Asp Val>

340 350 360 370 380
CCC CCC GAG GAG CCC CAG CTC TCC TGC TTC CGG AAG AGC CCC CTC AGC
Pro Pro Glu Glu Pro Gln Leu Ser Cys Phe Arg Lys Ser Pro Leu Ser>

390 400 410 420 430
AAT GTT GTT TGT GAG TGG GGT CCT CGG AGC ACC CCA TCC CTG ACG ACA
Asn Val Val Cys Glu Trp Gly Pro Arg Ser Thr Pro Ser Leu Thr Thr>

440 450 460 470 480
AAG GCT GTG CTC TTG GTG AGG AAG TTT CAG AAC AGT CCG GCC GAA GAC
Lys Ala Val Leu Leu Val Arg Lys Phe Gln Asn Ser Pro Ala Glu Asp>

490 500 510 520
TTC CAG GAG CCG TGC CAG TAT TCT CAG GAG TCC CAG AAG TTC TCC TGC
Phe Gln Glu Pro Cys Gln Tyr Ser Gln Glu Ser Gln Lys Phe Ser Cys>

530 540 550 560 570
CAG TTA GCA GTC CCG GAG GGA GAC AGC TCT TTC TAC ATA GTG TCC ATG
Gln Leu Ala Val Pro Glu Gly Asp Ser Ser Phe Tyr Ile Val Ser Met>

Figure 24B

```
 580       590       600       610       620
TGC GTC GCC AGT AGT GTC GGG AGC AAG TTC AGC AAA ACT CAA ACC TTT
Cys Val Ala Ser Ser Val Gly Ser Lys Phe Ser Lys Thr Gln Thr Phe>

   630       640       650       660       670
CAG GGT TGT GGA ATC TTG CAG CCT GAT CCG CCT GCC AAC ATC ACA GTC
Gln Gly Cys Gly Ile Leu Gln Pro Asp Pro Pro Ala Asn Ile Thr Val>

     680       690       700       710       720
ACT GCC GTG GCC AGA AAC CCC CGC TGG CTC AGT GTC ACC TGG CAA GAC
Thr Ala Val Ala Arg Asn Pro Arg Trp Leu Ser Val Thr Trp Gln Asp>

       730       740       750       760
CCC CAC TCC TGG AAC TCA TCT TTC TAC AGA CTA CGG TTT GAG CTC AGA
Pro His Ser Trp Asn Ser Ser Phe Tyr Arg Leu Arg Phe Glu Leu Arg>

770       780       790       800       810
TAT CGG GCT GAA CGG TCA AAG ACA TTC ACA ACA TGG ATG GTC AAG GAC
Tyr Arg Ala Glu Arg Ser Lys Thr Phe Thr Thr Trp Met Val Lys Asp>

  820       830       840       850       860
CTC CAG CAT CAC TGT GTC ATC CAC GAC GCC TGG AGC GGC CTG AGG CAC
Leu Gln His His Cys Val Ile His Asp Ala Trp Ser Gly Leu Arg His>

    870       880       890       900       910
GTG GTG CAG CTT CGT GCC CAG GAG GAG TTC GGG CAA GGC GAG TGG AGC
Val Val Gln Leu Arg Ala Gln Glu Glu Phe Gly Gln Gly Glu Trp Ser>

      920       930       940       950       960
GAG TGG AGC CCG GAG GCC ATG GGC ACG CCT TGG ACA GAA TCC AGG AGT
Glu Trp Ser Pro Glu Ala Met Gly Thr Pro Trp Thr Glu Ser Arg Ser>

        970       980       990      1000
CCT CCA GCT GAG AAC GAG GTG TCC ACC CCC ATG ACC GGT GGC GCG CCT
Pro Pro Ala Glu Asn Glu Val Ser Thr Pro Met Thr Gly Gly Ala Pro>

1010      1020      1030      1040      1050
TCA GGT GCT CAG CTG GAA CTT CTA GAC CCA TGT GGT TAT ATC AGT CCT
Ser Gly Ala Gln Leu Glu Leu Leu Asp Pro Cys Gly Tyr Ile Ser Pro>

  1060      1070      1080      1090      1100
GAA TCT CCA GTT GTA CAA CTT CAT TCT AAT TTC ACT GCA GTT TGT GTG
Glu Ser Pro Val Val Gln Leu His Ser Asn Phe Thr Ala Val Cys Val>

    1110      1120      1130      1140      1150
CTA AAG GAA AAA TGT ATG GAT TAT TTT CAT GTA AAT GCT AAT TAC ATT
Leu Lys Glu Lys Cys Met Asp Tyr Phe His Val Asn Ala Asn Tyr Ile>

      1160      1170      1180      1190      1200
```

Figure 24C

```
GTC TGG AAA ACA AAC CAT TTT ACT ATT CCT AAG GAG CAA TAT ACT ATC
Val Trp Lys Thr Asn His Phe Thr Ile Pro Lys Glu Gln Tyr Thr Ile>

        1210          1220          1230          1240
ATA AAT AGA ACA GCA TCC AGT GTC ACC TTT ACA GAT ATA GCT TCA TTA
Ile Asn Arg Thr Ala Ser Ser Val Thr Phe Thr Asp Ile Ala Ser Leu>

1250        1260          1270          1280          1290
AAT ATT CAG CTC ACT TGC AAC ATT CTT ACA TTT GGA CAG CTT GAA CAG
Asn Ile Gln Leu Thr Cys Asn Ile Leu Thr Phe Gly Gln Leu Glu Gln>

 1300          1310          1320          1330          1340
AAT GTT TAT GGA ATC ACA ATA ATT TCA GGC TTG CCT CCA GAA AAA CCT
Asn Val Tyr Gly Ile Thr Ile Ile Ser Gly Leu Pro Pro Glu Lys Pro>

   1350          1360          1370          1380          1390
AAA AAT TTG AGT TGC ATT GTG AAC GAG GGG AAG AAA ATG AGG TGT GAG
Lys Asn Leu Ser Cys Ile Val Asn Glu Gly Lys Lys Met Arg Cys Glu>

      1400          1410          1420          1430          1440
TGG GAT GGT GGA AGG GAA ACA CAC TTG GAG ACA AAC TTC ACT TTA AAA
Trp Asp Gly Gly Arg Glu Thr His Leu Glu Thr Asn Phe Thr Leu Lys>

         1450          1460          1470          1480
TCT GAA TGG GCA ACA CAC AAG TTT GCT GAT TGC AAA GCA AAA CGT GAC
Ser Glu Trp Ala Thr His Lys Phe Ala Asp Cys Lys Ala Lys Arg Asp>

1490        1500          1510          1520          1530
ACC CCC ACC TCA TGC ACT GTT GAT TAT TCT ACT GTG TAT TTT GTC AAC
Thr Pro Thr Ser Cys Thr Val Asp Tyr Ser Thr Val Tyr Phe Val Asn>

 1540          1550          1560          1570          1580
ATT GAA GTC TGG GTA GAA GCA GAG AAT GCC CTT GGG AAG GTT ACA TCA
Ile Glu Val Trp Val Glu Ala Glu Asn Ala Leu Gly Lys Val Thr Ser>

   1590          1600          1610          1620          1630
GAT CAT ATC AAT TTT GAT CCT GTA TAT AAA GTG AAG CCC AAT CCG CCA
Asp His Ile Asn Phe Asp Pro Val Tyr Lys Val Lys Pro Asn Pro Pro>

      1640          1650          1660          1670          1680
CAT AAT TTA TCA GTG ATC AAC TCA GAG GAA CTG TCT AGT ATC TTA AAA
His Asn Leu Ser Val Ile Asn Ser Glu Glu Leu Ser Ser Ile Leu Lys>

         1690          1700          1710          1720
TTG ACA TGG ACC AAC CCA AGT ATT AAG AGT GTT ATA ATA CTA AAA TAT
Leu Thr Trp Thr Asn Pro Ser Ile Lys Ser Val Ile Ile Leu Lys Tyr>

1730        1740          1750          1760          1770
AAC ATT CAA TAT AGG ACC AAA GAT GCC TCA ACT TGG AGC CAG ATT CCT
Asn Ile Gln Tyr Arg Thr Lys Asp Ala Ser Thr Trp Ser Gln Ile Pro>
```

Figure 24D

```
 1780       1790       1800       1810       1820
   *    *    *    *    *    *    *    *    *
CCT GAA GAC ACA GCA TCC ACC CGA TCT TCA TTC ACT GTC CAA GAC CTT
Pro Glu Asp Thr Ala Ser Thr Arg Ser Ser Phe Thr Val Gln Asp Leu>

   1830       1840       1850       1860       1870
 *    *    *    *    *    *    *    *    *    *
AAA CCT TTT ACA GAA TAT GTG TTT AGG ATT CGC TGT ATG AAG GAA GAT
Lys Pro Phe Thr Glu Tyr Val Phe Arg Ile Arg Cys Met Lys Glu Asp>

     1880       1890       1900       1910       1920
 *    *    *    *    *    *    *    *    *    *
GGT AAG GGA TAC TGG AGT GAC TGG AGT GAA GAA GCA AGT GGG ATC ACC
Gly Lys Gly Tyr Trp Ser Asp Trp Ser Glu Glu Ala Ser Gly Ile Thr>

        1930       1940       1950       1960
    *    *    *    *    *    *    *    *    *
TAT GAA GAT AGA CCA TCT AAA GCA CCA AGT TTC TGG TAT AAA ATA GAT
Tyr Glu Asp Arg Pro Ser Lys Ala Pro Ser Phe Trp Tyr Lys Ile Asp>

1970       1980       1990       2000       2010
 *    *    *    *    *    *    *    *    *    *
CCA TCC CAT ACT CAA GGC TAC AGA ACT GTA CAA CTC GTG TGG AAG ACA
Pro Ser His Thr Gln Gly Tyr Arg Thr Val Gln Leu Val Trp Lys Thr>

 2020       2030       2040       2050       2060
    *    *    *    *    *    *    *    *    *
TTG CCT CCT TTT GAA GCC AAT GGA AAA ATC TTG GAT TAT GAA GTG ACT
Leu Pro Pro Phe Glu Ala Asn Gly Lys Ile Leu Asp Tyr Glu Val Thr>

   2070       2080       2090       2100       2110
 *    *    *    *    *    *    *    *    *    *
CTC ACA AGA TGG AAA TCA CAT TTA CAA AAT TAC ACA GTT AAT GCC ACA
Leu Thr Arg Trp Lys Ser His Leu Gln Asn Tyr Thr Val Asn Ala Thr>

     2120       2130       2140       2150       2160
 *    *    *    *    *    *    *    *    *    *
AAA CTG ACA GTA AAT CTC ACA AAT GAT CGC TAT CTA GCA ACC CTA ACA
Lys Leu Thr Val Asn Leu Thr Asn Asp Arg Tyr Leu Ala Thr Leu Thr>

        2170       2180       2190       2200
    *    *    *    *    *    *    *    *    *
GTA AGA AAT CTT GTT GGC AAA TCA GAT GCA GCT GTT TTA ACT ATC CCT
Val Arg Asn Leu Val Gly Lys Ser Asp Ala Ala Val Leu Thr Ile Pro>

2210       2220       2230       2240       2250
 *    *    *    *    *    *    *    *    *    *
GCC TGT GAC TTT CAA GCT ACT CAC CCT GTA ATG GAT CTT AAA GCA TTC
Ala Cys Asp Phe Gln Ala Thr His Pro Val Met Asp Leu Lys Ala Phe>

 2260       2270       2280       2290       2300
    *    *    *    *    *    *    *    *    *
CCC AAA GAT AAC ATG CTT TGG GTG GAA TGG ACT ACT CCA AGG GAA TCT
Pro Lys Asp Asn Met Leu Trp Val Glu Trp Thr Thr Pro Arg Glu Ser>

   2310       2320       2330       2340       2350
 *    *    *    *    *    *    *    *    *    *
GTA AAG AAA TAT ATA CTT GAG TGG TGT GTG TTA TCA GAT AAA GCA CCC
Val Lys Lys Tyr Ile Leu Glu Trp Cys Val Leu Ser Asp Lys Ala Pro>

     2360       2370       2380       2390       2400
```

Figure 24E

TGT ATC ACA GAC TGG CAA CAA GAA GAT GGT ACC GTG CAT CGC ACC TAT
Cys Ile Thr Asp Trp Gln Gln Glu Asp Gly Thr Val His Arg Thr Tyr>

2410 2420 2430 2440

TTA AGA GGG AAC TTA GCA GAG AGC AAA TGC TAT TTG ATA ACA GTT ACT
Leu Arg Gly Asn Leu Ala Glu Ser Lys Cys Tyr Leu Ile Thr Val Thr>

2450 2460 2470 2480 2490

CCA GTA TAT GCT GAT GGA CCA GGA AGC CCT GAA TCC ATA AAG GCA TAC
Pro Val Tyr Ala Asp Gly Pro Gly Ser Pro Glu Ser Ile Lys Ala Tyr>

2500 2510 2520 2530 2540

CTT AAA CAA GCT CCA CCT TCC AAA GGA CCT ACT GTT CGG ACA AAA AAA
Leu Lys Gln Ala Pro Pro Ser Lys Gly Pro Thr Val Arg Thr Lys Lys>

2550 2560 2570 2580 2590

GTA GGG AAA AAC GAA GCT GTC TTA GAG TGG GAC CAA CTT CCT GTT GAT
Val Gly Lys Asn Glu Ala Val Leu Glu Trp Asp Gln Leu Pro Val Asp>

2600 2610 2620 2630 2640

GTT CAG AAT GGA TTT ATC AGA AAT TAT ACT ATA TTT TAT AGA ACC ATC
Val Gln Asn Gly Phe Ile Arg Asn Tyr Thr Ile Phe Tyr Arg Thr Ile>

2650 2660 2670 2680

ATT GGA AAT GAA ACT GCT GTG AAT GTG GAT TCT TCC CAC ACA GAA TAT
Ile Gly Asn Glu Thr Ala Val Asn Val Asp Ser Ser His Thr Glu Tyr>

2690 2700 2710 2720 2730

ACA TTG TCC TCT TTG ACT AGT GAC ACA TTG TAC ATG GTA CGA ATG GCA
Thr Leu Ser Ser Leu Thr Ser Asp Thr Leu Tyr Met Val Arg Met Ala>

2740 2750 2760 2770 2780

GCA TAC ACA GAT GAA GGT GGG AAG GAT GGT CCA GAA TTC ACT TTT ACT
Ala Tyr Thr Asp Glu Gly Gly Lys Asp Gly Pro Glu Phe Thr Phe Thr>

2790 2800 2810 2820 2830

ACC CCA AAG TTT GCT CAA GGA GAA ATT GAA TCC GGG GGC GAC AAA ACT
Thr Pro Lys Phe Ala Gln Gly Glu Ile Glu Ser Gly Gly Asp Lys Thr>

2840 2850 2860 2870 2880

CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA
His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser>

2890 2900 2910 2920

GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg>

2930 2940 2950 2960 2970

ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT

igure 24F

Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro>

2980 2990 3000 3010 3020

GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC
Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala>

3030 3040 3050 3060 3070

AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC
Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val>

3080 3090 3100 3110 3120

AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC
Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr>

3130 3140 3150 3160

AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC
Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>

3170 3180 3190 3200 3210

ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu>

3220 3230 3240 3250 3260

CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC
Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys>

3270 3280 3290 3300 3310

CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser>

3320 3330 3340 3350 3360

AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp>

3370 3380 3390 3400

TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser>

3410 3420 3430 3440 3450

AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala>

3460 3470 3480 3490 3500

CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA
Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys>

TGA
***>

Figure 25A

```
         10          20          30          40
ATG GTG GCC GTC GGC TGC GCG CTG CTG GCT GCC CTG CTG GCC GCG CCG
Met Val Ala Val Gly Cys Ala Leu Leu Ala Ala Leu Leu Ala Ala Pro>

 50          60          70          80          90
GGA GCG GCG CTG GCC CCA AGG CGT TGC CCT GCG CAG GAG GTG GCA AGA
Gly Ala Ala Leu Ala Pro Arg Arg Cys Pro Ala Gln Glu Val Ala Arg>

   100         110         120         130         140
GGC GTG CTG ACC AGT CTG CCA GGA GAC AGC GTG ACT CTG ACC TGC CCG
Gly Val Leu Thr Ser Leu Pro Gly Asp Ser Val Thr Leu Thr Cys Pro>

     150         160         170         180         190
GGG GTA GAG CCG GAA GAC AAT GCC ACT GTT CAC TGG GTG CTC AGG AAG
Gly Val Glu Pro Glu Asp Asn Ala Thr Val His Trp Val Leu Arg Lys>

       200         210         220         230         240
CCG GCT GCA GGC TCC CAC CCC AGC AGA TGG GCT GGC ATG GGA AGG AGG
Pro Ala Ala Gly Ser His Pro Ser Arg Trp Ala Gly Met Gly Arg Arg>

         250         260         270         280
CTG CTG CTG AGG TCG GTG CAG CTC CAC GAC TCT GGA AAC TAT TCA TGC
Leu Leu Leu Arg Ser Val Gln Leu His Asp Ser Gly Asn Tyr Ser Cys>

290         300         310         320         330
TAC CGG GCC GGC CGC CCA GCT GGG ACT GTG CAC TTG CTG GTG GAT GTT
Tyr Arg Ala Gly Arg Pro Ala Gly Thr Val His Leu Leu Val Asp Val>

  340         350         360         370         380
CCC CCC GAG GAG CCC CAG CTC TCC TGC TTC CGG AAG AGC CCC CTC AGC
Pro Pro Glu Glu Pro Gln Leu Ser Cys Phe Arg Lys Ser Pro Leu Ser>

    390         400         410         420         430
AAT GTT GTT TGT GAG TGG GGT CCT CGG AGC ACC CCA TCC CTG ACG ACA
Asn Val Val Cys Glu Trp Gly Pro Arg Ser Thr Pro Ser Leu Thr Thr>

      440         450         460         470         480
AAG GCT GTG CTC TTG GTG AGG AAG TTT CAG AAC AGT CCG GCC GAA GAC
Lys Ala Val Leu Leu Val Arg Lys Phe Gln Asn Ser Pro Ala Glu Asp>

        490         500         510         520
TTC CAG GAG CCG TGC CAG TAT TCC CAG GAG TCC CAG AAG TTC TCC TGC
Phe Gln Glu Pro Cys Gln Tyr Ser Gln Glu Ser Gln Lys Phe Ser Cys>

530         540         550         560         570
CAG TTA GCA GTC CCG GAG GGA GAC AGC TCT TTC TAC ATA GTG TCC ATG
Gln Leu Ala Val Pro Glu Gly Asp Ser Ser Phe Tyr Ile Val Ser Met>
```

Figure 25B

```
 580       590       600       610       620
   *    *    *    *    *    *    *    *    *
TGC GTC GCC AGT AGT GTC GGG AGC AAG TTC AGC AAA ACT CAA ACC TTT
Cys Val Ala Ser Ser Val Gly Ser Lys Phe Ser Lys Thr Gln Thr Phe>

   630       640       650       660       670
 *    *    *    *    *    *    *    *    *    *
CAG GGT TGT GGA ATC TTG CAG CCT GAT CCG CCT GCC AAC ATC ACA GTC
Gln Gly Cys Gly Ile Leu Gln Pro Asp Pro Pro Ala Asn Ile Thr Val>

      680       690       700       710       720
 *    *    *    *    *    *    *    *    *    *
ACT GCC GTG GCC AGA AAC CCC CGC TGG CTC AGT GTC ACC TGG CAA GAC
Thr Ala Val Ala Arg Asn Pro Arg Trp Leu Ser Val Thr Trp Gln Asp>

         730       740       750       760
   *    *    *    *    *    *    *    *    *
CCC CAC TCC TGG AAC TCA TCT TTC TAC AGA CTA CGG TTT GAG CTC AGA
Pro His Ser Trp Asn Ser Ser Phe Tyr Arg Leu Arg Phe Glu Leu Arg>

770       780       790       800       810
 *    *    *    *    *    *    *    *    *    *
TAT CGG GCT GAA CGG TCA AAG ACA TTC ACA ACA TGG ATG GTC AAG GAC
Tyr Arg Ala Glu Arg Ser Lys Thr Phe Thr Thr Trp Met Val Lys Asp>

  820       830       840       850       860
   *    *    *    *    *    *    *    *    *
CTC CAG CAT CAC TGT GTC ATC CAC GAC GCC TGG AGC GGC CTG AGG CAC
Leu Gln His His Cys Val Ile His Asp Ala Trp Ser Gly Leu Arg His>

     870       880       890       900       910
 *    *    *    *    *    *    *    *    *    *
GTG GTG CAG CTT CGT GCC CAG GAG GAG TTC GGG CAA GGC GAG TGG AGC
Val Val Gln Leu Arg Ala Gln Glu Glu Phe Gly Gln Gly Glu Trp Ser>

        920       930       940       950       960
 *    *    *    *    *    *    *    *    *    *
GAG TGG AGC CCG GAG GCC ATG GGC ACG CCT TGG ACA GAA TCG CGA TCG
Glu Trp Ser Pro Glu Ala Met Gly Thr Pro Trp Thr Glu Ser Arg Ser>

           970       980       990      1000
   *    *    *    *    *    *    *    *    *
CCT CCA GCT GAG AAC GAG GTG TCC ACC CCC ATG GAA CTT CTA GAC CCA
Pro Pro Ala Glu Asn Glu Val Ser Thr Pro Met Glu Leu Leu Asp Pro>

1010      1020      1030      1040      1050
 *    *    *    *    *    *    *    *    *    *
TGT GGT TAT ATC AGT CCT GAA TCT CCA GTT GTA CAA CTT CAT TCT AAT
Cys Gly Tyr Ile Ser Pro Glu Ser Pro Val Val Gln Leu His Ser Asn>

 1060      1070      1080      1090      1100
   *    *    *    *    *    *    *    *    *
TTC ACT GCA GTT TGT GTG CTA AAG GAA AAA TGT ATG GAT TAT TTT CAT
Phe Thr Ala Val Cys Val Leu Lys Glu Lys Cys Met Asp Tyr Phe His>

    1110      1120      1130      1140      1150
 *    *    *    *    *    *    *    *    *    *
GTA AAT GCT AAT TAC ATT GTC TGG AAA ACA AAC CAT TTT ACT ATT CCT
Val Asn Ala Asn Tyr Ile Val Trp Lys Thr Asn His Phe Thr Ile Pro>

       1160      1170      1180      1190      1200
 *    *    *    *    *    *    *    *    *    *
```

Figure 25C

AAG GAG CAA TAT ACT ATC ATA AAC AGA ACA GCA TCC AGT GTC ACC TTT
Lys Glu Gln Tyr Thr Ile Ile Asn Arg Thr Ala Ser Ser Val Thr Phe>

1210 1220 1230 1240

ACA GAT ATA GCT TCA TTA AAT ATT CAG CTC ACT TGC AAC ATT CTT ACA
Thr Asp Ile Ala Ser Leu Asn Ile Gln Leu Thr Cys Asn Ile Leu Thr>

1250 1260 1270 1280 1290

TTC GGA CAG CTT GAA CAG AAT GTT TAT GGA ATC ACA ATA ATT TCA GGC
Phe Gly Gln Leu Glu Gln Asn Val Tyr Gly Ile Thr Ile Ile Ser Gly>

1300 1310 1320 1330 1340

TTG CCT CCA GAA AAA CCT AAA AAT TTG AGT TGC ATT GTG AAC GAG GGG
Leu Pro Pro Glu Lys Pro Lys Asn Leu Ser Cys Ile Val Asn Glu Gly>

1350 1360 1370 1380 1390

AAG AAA ATG AGG TGT GAG TGG GAT GGT GGA AGG GAA ACA CAC TTG GAG
Lys Lys Met Arg Cys Glu Trp Asp Gly Gly Arg Glu Thr His Leu Glu>

1400 1410 1420 1430 1440

ACA AAC TTC ACT TTA AAA TCT GAA TGG GCA ACA CAC AAG TTT GCT GAT
Thr Asn Phe Thr Leu Lys Ser Glu Trp Ala Thr His Lys Phe Ala Asp>

1450 1460 1470 1480

TGC AAA GCA AAA CGT GAC ACC CCC ACC TCA TGC ACT GTT GAT TAT TCT
Cys Lys Ala Lys Arg Asp Thr Pro Thr Ser Cys Thr Val Asp Tyr Ser>

1490 1500 1510 1520 1530

ACT GTG TAT TTT GTC AAC ATT GAA GTC TGG GTA GAA GCA GAG AAT GCC
Thr Val Tyr Phe Val Asn Ile Glu Val Trp Val Glu Ala Glu Asn Ala>

1540 1550 1560 1570 1580

CTT GGG AAG GTT ACA TCA GAT CAT ATC AAT TTT GAT CCT GTA TAT AAA
Leu Gly Lys Val Thr Ser Asp His Ile Asn Phe Asp Pro Val Tyr Lys>

1590 1600 1610 1620 1630

GTG AAG CCC AAT CCG CCA CAT AAT TTA TCA GTG ATC AAC TCA GAG GAA
Val Lys Pro Asn Pro Pro His Asn Leu Ser Val Ile Asn Ser Glu Glu>

1640 1650 1660 1670 1680

CTG TCT AGT ATC TTA AAA TTG ACA TGG ACC AAC CCA AGT ATT AAG AGT
Leu Ser Ser Ile Leu Lys Leu Thr Trp Thr Asn Pro Ser Ile Lys Ser>

1690 1700 1710 1720

GTT ATA ATA CTA AAA TAC AAC ATT CAA TAT AGG ACC AAA GAT GCC TCA
Val Ile Ile Leu Lys Tyr Asn Ile Gln Tyr Arg Thr Lys Asp Ala Ser>

1730 1740 1750 1760 1770

ACT TGG AGC CAG ATT CCT CCT GAA GAC ACA GCA TCC ACC CGA TCT TCA
Thr Trp Ser Gln Ile Pro Pro Glu Asp Thr Ala Ser Thr Arg Ser Ser>

Figure 25D

```
1780      1790      1800      1810      1820
TTC ACT GTG CAA GAC CTT AAA CCT TTT ACA GAA TAT GTG TTT AGG ATT
Phe Thr Val Gln Asp Leu Lys Pro Phe Thr Glu Tyr Val Phe Arg Ile>

1830      1840      1850      1860      1870
CGC TGT ATG AAG GAA GAT GGT AAG GGA TAC TGG AGT GAC TGG AGT GAA
Arg Cys Met Lys Glu Asp Gly Lys Gly Tyr Trp Ser Asp Trp Ser Glu>

1880      1890      1900      1910      1920
GAA GCA AGT GGG ATC ACC TAT GAA GAT AGA CCA TCT AAA GCA CCA AGT
Glu Ala Ser Gly Ile Thr Tyr Glu Asp Arg Pro Ser Lys Ala Pro Ser>

1930      1940      1950      1960
TTC TGG TAT AAA ATA GAT CCA TCC CAT ACT CAA GGC TAC AGA ACT GTA
Phe Trp Tyr Lys Ile Asp Pro Ser His Thr Gln Gly Tyr Arg Thr Val>

1970      1980      1990      2000      2010
CAA CTC GTG TGG AAG ACA TTG CCT CCT TTT GAA GCC AAT GGA AAA ATC
Gln Leu Val Trp Lys Thr Leu Pro Pro Phe Glu Ala Asn Gly Lys Ile>

2020      2030      2040      2050      2060
TTG GAT TAT GAA GTG ACT CTC ACA AGA TGG AAA TCA CAT TTA CAA AAT
Leu Asp Tyr Glu Val Thr Leu Thr Arg Trp Lys Ser His Leu Gln Asn>

2070      2080      2090      2100      2110
TAC ACA GTT AAT GCC ACA AAA CTG ACA GTA AAT CTC ACA AAT GAT CGC
Tyr Thr Val Asn Ala Thr Lys Leu Thr Val Asn Leu Thr Asn Asp Arg>

2120      2130      2140      2150      2160
TAT CTA GCA ACC CTA ACA GTA AGA AAT CTT GTT GGC AAA TCA GAT GCA
Tyr Leu Ala Thr Leu Thr Val Arg Asn Leu Val Gly Lys Ser Asp Ala>

2170      2180      2190      2200
GCT GTT TTA ACT ATC CCT GCC TGT GAC TTT CAA GCT ACT CAC CCT GTA
Ala Val Leu Thr Ile Pro Ala Cys Asp Phe Gln Ala Thr His Pro Val>

2210      2220      2230      2240      2250
ATG GAT CTT AAA GCA TTC CCC AAA GAT AAC ATG CTT TGG GTG GAA TGG
Met Asp Leu Lys Ala Phe Pro Lys Asp Asn Met Leu Trp Val Glu Trp>

2260      2270      2280      2290      2300
ACT ACT CCA AGG GAA TCT GTA AAG AAA TAT ATA CTT GAG TGG TGT GTG
Thr Thr Pro Arg Glu Ser Val Lys Lys Tyr Ile Leu Glu Trp Cys Val>

2310      2320      2330      2340      2350
TTA TCA GAT AAA GCA CCC TGT ATC ACA GAC TGG CAA CAA GAA GAT GGT
Leu Ser Asp Lys Ala Pro Cys Ile Thr Asp Trp Gln Gln Glu Asp Gly>

2360      2370      2380      2390      2400
```

Figure 25E

ACC GTG CAT CGC ACC TAT TTA AGA GGG AAC TTA GCA GAG AGC AAA TGC
Thr Val His Arg Thr Tyr Leu Arg Gly Asn Leu Ala Glu Ser Lys Cys>

2410 2420 2430 2440

TAT TTG ATA ACA GTT ACT CCA GTA TAT GCT GAT GGA CCA GGA AGC CCT
Tyr Leu Ile Thr Val Thr Pro Val Tyr Ala Asp Gly Pro Gly Ser Pro>

2450 2460 2470 2480 2490

GAA TCC ATA AAG GCA TAC CTT AAA CAA GCT CCA CCT TCC AAA GGA CCT
Glu Ser Ile Lys Ala Tyr Leu Lys Gln Ala Pro Pro Ser Lys Gly Pro>

2500 2510 2520 2530 2540

ACT GTT CGG ACA AAA AAA GTA GGG AAA AAC GAA GCT GTC TTA GAG TGG
Thr Val Arg Thr Lys Lys Val Gly Lys Asn Glu Ala Val Leu Glu Trp>

2550 2560 2570 2580 2590

GAC CAA CTT CCT GTT GAT GTT CAG AAT GGA TTT ATC AGA AAT TAT ACT
Asp Gln Leu Pro Val Asp Val Gln Asn Gly Phe Ile Arg Asn Tyr Thr>

2600 2610 2620 2630 2640

ATA TTT TAT AGA ACC ATC ATT GGA AAT GAA ACT GCT GTG AAT GTG GAT
Ile Phe Tyr Arg Thr Ile Ile Gly Asn Glu Thr Ala Val Asn Val Asp>

2650 2660 2670 2680

TCT TCC CAC ACA GAA TAT ACA TTG TCC TCT TTG ACT AGT GAC ACA TTG
Ser Ser His Thr Glu Tyr Thr Leu Ser Ser Leu Thr Ser Asp Thr Leu>

2690 2700 2710 2720 2730

TAC ATG GTA CGA ATG GCA GCA TAC ACA GAT GAA GGT GGG AAG GAT GGT
Tyr Met Val Arg Met Ala Ala Tyr Thr Asp Glu Gly Gly Lys Asp Gly>

2740 2750 2760 2770 2780

CCA GAA TTC ACT TTT ACT ACC CCA AAG TTT GCT CAA GGA GAA ATT GAA
Pro Glu Phe Thr Phe Thr Thr Pro Lys Phe Ala Gln Gly Glu Ile Glu>

2790 2800 2810 2820 2830

TCC GGG GGC GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA
Ser Gly Gly Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu>

2840 2850 2860 2870 2880

CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC
Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp>

2890 2900 2910 2920

ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC
Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp>

2930 2940 2950 2960 2970

GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC

Figure 25F

Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly>

2980 2990 3000 3010 3020

GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC
Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn>

3030 3040 3050 3060 3070

AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG
Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp>

3080 3090 3100 3110 3120

CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA
Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro>

3130 3140 3150 3160

GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA
Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu>

3170 3180 3190 3200 3210

CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC
Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn>

3220 3230 3240 3250 3260

CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC
Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile>

3270 3280 3290 3300 3310

GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC
Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr>

3320 3330 3340 3350 3360

ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG
Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys>

3370 3380 3390 3400

CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC
Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys>

3410 3420 3430 3440 3450

TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC
Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu>

3460 3470

TCC CTG TCT CCG GGT AAA TGA
Ser Leu Ser Pro Gly Lys ***.

Figure 26A

```
         10         20         30         40
ATG GTG CTT CTG TGG TGT GTA GTG AGT CTC TAC TTT TAT GGA ATC CTG
Met Val Leu Leu Trp Cys Val Val Ser Leu Tyr Phe Tyr Gly Ile Leu>

 50         60         70         80         90
CAA AGT GAT GCC TCA GAA CGC TGC GAT GAC TGG GGA CTA GAC ACC ATG
Gln Ser Asp Ala Ser Glu Arg Cys Asp Asp Trp Gly Leu Asp Thr Met>

  100        110        120        130        140
AGG CAA ATC CAA GTG TTT GAA GAT GAG CCA GCT CGC ATC AAG TGC CCA
Arg Gln Ile Gln Val Phe Glu Asp Glu Pro Ala Arg Ile Lys Cys Pro>

   150        160        170        180        190
CTC TTT GAA CAC TTC TTG AAA TTC AAC TAC AGC ACA GCC CAT TCA GCT
Leu Phe Glu His Phe Leu Lys Phe Asn Tyr Ser Thr Ala His Ser Ala>

     200        210        220        230        240
GGC CTT ACT CTG ATC TGG TAT TGG ACT AGG CAG GAC CGG GAC CTT GAG
Gly Leu Thr Leu Ile Trp Tyr Trp Thr Arg Gln Asp Arg Asp Leu Glu>

      250        260        270        280
GAG CCA ATT AAC TTC CGC CTC CCC GAG AAC CGC ATT AGT AAG GAG AAA
Glu Pro Ile Asn Phe Arg Leu Pro Glu Asn Arg Ile Ser Lys Glu Lys>

290        300        310        320        330
GAT GTG CTG TGG TTC CGG CCC ACT CTC CTC AAT GAC ACT GGC AAC TAT
Asp Val Leu Trp Phe Arg Pro Thr Leu Leu Asn Asp Thr Gly Asn Tyr>

  340        350        360        370        380
ACC TGC ATG TTA AGG AAC ACT ACA TAT TGC AGC AAA GTT GCA TTT CCC
Thr Cys Met Leu Arg Asn Thr Thr Tyr Cys Ser Lys Val Ala Phe Pro>

   390        400        410        420        430
TTG GAA GTT GTT CAA AAA GAC AGC TGT TTC AAT TCC CCC ATG AAA CTC
Leu Glu Val Val Gln Lys Asp Ser Cys Phe Asn Ser Pro Met Lys Leu>

    440        450        460        470        480
CCA GTG CAT AAA CTG TAT ATA GAA TAT GGC ATT CAG AGG ATC ACT TGT
Pro Val His Lys Leu Tyr Ile Glu Tyr Gly Ile Gln Arg Ile Thr Cys>

      490        500        510        520
CCA AAT GTA GAT GGA TAT TTT CCT TCT AGT GTC AAA CCG ACT ATC ACT
Pro Asn Val Asp Gly Tyr Phe Pro Ser Ser Val Lys Pro Thr Ile Thr>

530        540        550        560        570
TGG TAT ATG GGC TGT TAT AAA ATA CAG AAT TTT AAT AAT GTA ATA CCT
Trp Tyr Met Gly Cys Tyr Lys Ile Gln Asn Phe Asn Asn Val Ile Pro>
```

Figure 26B

```
 580        590        600        610        620
GAA GGT ATG AAC TTG AGT TTC CTC ATT GCC TTA ATT TCA AAT AAT GGA
Glu Gly Met Asn Leu Ser Phe Leu Ile Ala Leu Ile Ser Asn Asn Gly>

 630        640        650        660        670
AAT TAC ACA TGT GTT GTT ACA TAT CCA GAA AAT GGA CGT ACG TTT CAT
Asn Tyr Thr Cys Val Val Thr Tyr Pro Glu Asn Gly Arg Thr Phe His>

 680        690        700        710        720
CTC ACC AGG ACT CTG ACT GTA AAG GTA GTA GGC TCT CCA AAA AAT GCA
Leu Thr Arg Thr Leu Thr Val Lys Val Val Gly Ser Pro Lys Asn Ala>

 730        740        750        760
GTG CCC CCT GTG ATC CAT TCA CCT AAT GAT CAT GTG GTC TAT GAG AAA
Val Pro Pro Val Ile His Ser Pro Asn Asp His Val Val Tyr Glu Lys>

770        780        790        800        810
GAA CCA GGA GAG GAG CTA CTC ATT CCC TGT ACG GTC TAT TTT AGT TTT
Glu Pro Gly Glu Glu Leu Leu Ile Pro Cys Thr Val Tyr Phe Ser Phe>

 820        830        840        850        860
CTG ATG GAT TCT CGC AAT GAG GTT TGG TGG ACC ATT GAT GGA AAA AAA
Leu Met Asp Ser Arg Asn Glu Val Trp Trp Thr Ile Asp Gly Lys Lys>

 870        880        890        900        910
CCT GAT GAC ATC ACT ATT GAT GTC ACC ATT AAC GAA AGT ATA AGT CAT
Pro Asp Asp Ile Thr Ile Asp Val Thr Ile Asn Glu Ser Ile Ser His>

 920        930        940        950        960
AGT AGA ACA GAA GAT GAA ACA AGA ACT CAG ATT TTG AGC ATC AAG AAA
Ser Arg Thr Glu Asp Glu Thr Arg Thr Gln Ile Leu Ser Ile Lys Lys>

 970        980        990        1000
GTT ACC TCT GAG GAT CTC AAG CGC AGC TAT GTC TGT CAT GCT AGA AGT
Val Thr Ser Glu Asp Leu Lys Arg Ser Tyr Val Cys His Ala Arg Ser>

1010       1020       1030       1040       1050
GCC AAA GGC GAA GTT GCC AAA GCA GCC AAG GTG AAG CAG AAA GTG CCA
Ala Lys Gly Glu Val Ala Lys Ala Ala Lys Val Lys Gln Lys Val Pro>

1060       1070       1080       1090       1100
GCT CCA AGA TAC ACA GTG TCC GGT GGC GCG CCT ATG CTG AGC GAG GCT
Ala Pro Arg Tyr Thr Val Ser Gly Gly Ala Pro Met Leu Ser Glu Ala>

1110       1120       1130       1140       1150
GAT AAA TGC AAG GAA CGT GAA GAA AAA ATA ATT TTA GTG TCA TCT GCA
Asp Lys Cys Lys Glu Arg Glu Glu Lys Ile Ile Leu Val Ser Ser Ala>

1160       1170       1180       1190       1200
```

Figure 26C

```
AAT GAA ATT GAT GTT CGT CCC TGT CCT CTT AAC CCA AAT GAA CAC AAA
Asn Glu Ile Asp Val Arg Pro Cys Pro Leu Asn Pro Asn Glu His Lys>

         1210          1220          1230          1240
   *      *       *      *      *      *      *      *      *
GGC ACT ATA ACT TGG TAT AAG GAT GAC AGC AAG ACA CCT GTA TCT ACA
Gly Thr Ile Thr Trp Tyr Lys Asp Asp Ser Lys Thr Pro Val Ser Thr>

1250         1260          1270          1280          1290
   *      *       *      *      *      *      *      *      *
GAA CAA GCC TCC AGG ATT CAT CAA CAC AAA GAG AAA CTT TGG TTT GTT
Glu Gln Ala Ser Arg Ile His Gln His Lys Glu Lys Leu Trp Phe Val>

 1300          1310          1320          1330          1340
   *      *       *      *      *      *      *      *      *
CCT GCT AAG GTG GAG GAT TCA GGA CAT TAC TAT TGC GTG GTA AGA AAT
Pro Ala Lys Val Glu Asp Ser Gly His Tyr Tyr Cys Val Val Arg Asn>

         1350          1360          1370          1380          1390
   *      *       *      *      *      *      *      *      *
TCA TCT TAC TGC CTC AGA ATT AAA ATA AGT GCA AAA TTT GTG GAG AAT
Ser Ser Tyr Cys Leu Arg Ile Lys Ile Ser Ala Lys Phe Val Glu Asn>

         1400          1410          1420          1430          1440
   *      *       *      *      *      *      *      *      *
GAG CCT AAC TTA TGT TAT AAT GCA CAA GCC ATA TTT AAG CAG AAA CTA
Glu Pro Asn Leu Cys Tyr Asn Ala Gln Ala Ile Phe Lys Gln Lys Leu>

            1450          1460          1470          1480
   *      *       *      *      *      *      *      *      *
CCC GTT GCA GGA GAC GGA GGA CTT GTG TGC CCT TAT ATG GAG TTT TTT
Pro Val Ala Gly Asp Gly Gly Leu Val Cys Pro Tyr Met Glu Phe Phe>

1490         1500          1510          1520          1530
   *      *       *      *      *      *      *      *      *
AAA AAT GAA AAT AAT GAG TTA CCT AAA TTA CAG TGG TAT AAG GAT TGC
Lys Asn Glu Asn Asn Glu Leu Pro Lys Leu Gln Trp Tyr Lys Asp Cys>

 1540          1550          1560          1570          1580
   *      *       *      *      *      *      *      *      *
AAA CCT CTA CTT CTT GAC AAT ATA CAC TTT AGT GGA GTC AAA GAT AGG
Lys Pro Leu Leu Leu Asp Asn Ile His Phe Ser Gly Val Lys Asp Arg>

         1590          1600          1610          1620          1630
   *      *       *      *      *      *      *      *      *
CTC ATC GTG ATG AAT GTG GCT GAA AAG CAT AGA GGG AAC TAT ACT TGT
Leu Ile Val Met Asn Val Ala Glu Lys His Arg Gly Asn Tyr Thr Cys>

            1640          1650          1660          1670          1680
   *      *       *      *      *      *      *      *      *
CAT GCA TCC TAC ACA TAC TTG GGC AAG CAA TAT CCT ATT ACC CGG GTA
His Ala Ser Tyr Thr Tyr Leu Gly Lys Gln Tyr Pro Ile Thr Arg Val>

            1690          1700          1710          1720
   *      *       *      *      *      *      *      *      *
ATA GAA TTT ATT ACT CTA GAG GAA AAC AAA CCC ACA AGG CCT GTG ATT
Ile Glu Phe Ile Thr Leu Glu Glu Asn Lys Pro Thr Arg Pro Val Ile>

1730         1740          1750          1760          1770
   *      *       *      *      *      *      *      *      *
GTG AGC CCA GCT AAT GAG ACA ATG GAA GTA GAC TTG GGA TCC CAG ATA
Val Ser Pro Ala Asn Glu Thr Met Glu Val Asp Leu Gly Ser Gln Ile>
```

Figure 26D

```
1780      1790      1800      1810      1820
CAA TTG ATC TGT AAT GTC ACC GGC CAG TTG AGT GAC ATT GCT TAC TGG
Gln Leu Ile Cys Asn Val Thr Gly Gln Leu Ser Asp Ile Ala Tyr Trp>

 1830      1840      1850      1860      1870
AAG TGG AAT GGG TCA GTA ATT GAT GAA GAT GAC CCA GTG CTA GGG GAA
Lys Trp Asn Gly Ser Val Ile Asp Glu Asp Asp Pro Val Leu Gly Glu>

  1880      1890      1900      1910      1920
GAC TAT TAC AGT GTG GAA AAT CCT GCA AAC AAA AGA AGG AGT ACC CTC
Asp Tyr Tyr Ser Val Glu Asn Pro Ala Asn Lys Arg Arg Ser Thr Leu>

   1930      1940      1950      1960
ATC ACA GTG CTT AAT ATA TCG GAA ATT GAG AGT AGA TTT TAT AAA CAT
Ile Thr Val Leu Asn Ile Ser Glu Ile Glu Ser Arg Phe Tyr Lys His>

1970      1980      1990      2000      2010
CCA TTT ACC TGT TTT GCC AAG AAT ACA CAT GGT ATA GAT GCA GCA TAT
Pro Phe Thr Cys Phe Ala Lys Asn Thr His Gly Ile Asp Ala Ala Tyr>

 2020      2030      2040      2050      2060
ATC CAG TTA ATA TAT CCA GTC ACT AAT TCC GGA GAC AAA ACT CAC ACA
Ile Gln Leu Ile Tyr Pro Val Thr Asn Ser Gly Asp Lys Thr His Thr>

  2070      2080      2090      2100      2110
TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe>

   2120      2130      2140      2150      2160
CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro>

     2170      2180      2190      2200
GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val>

2210      2220      2230      2240      2250
AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr>

 2260      2270      2280      2290      2300
AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val>

  2310      2320      2330      2340      2350
CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys>

   2360      2370      2380      2390      2400
```

Figure 26E

```
AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser>

        2410        2420        2430        2440
AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro>

2450        2460        2470        2480        2490
TCC CGG GAG GAG ATG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC
Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val>

 2500        2510        2520        2530        2540
AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly>

   2550        2560        2570        2580        2590
CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp>

      2600        2610        2620        2630        2640
GGC TCC TTC TTC CTC TAT AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp>

        2650        2660        2670        2680
CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His>

2690        2700        2710        2720        2730
AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
```

Figure 27

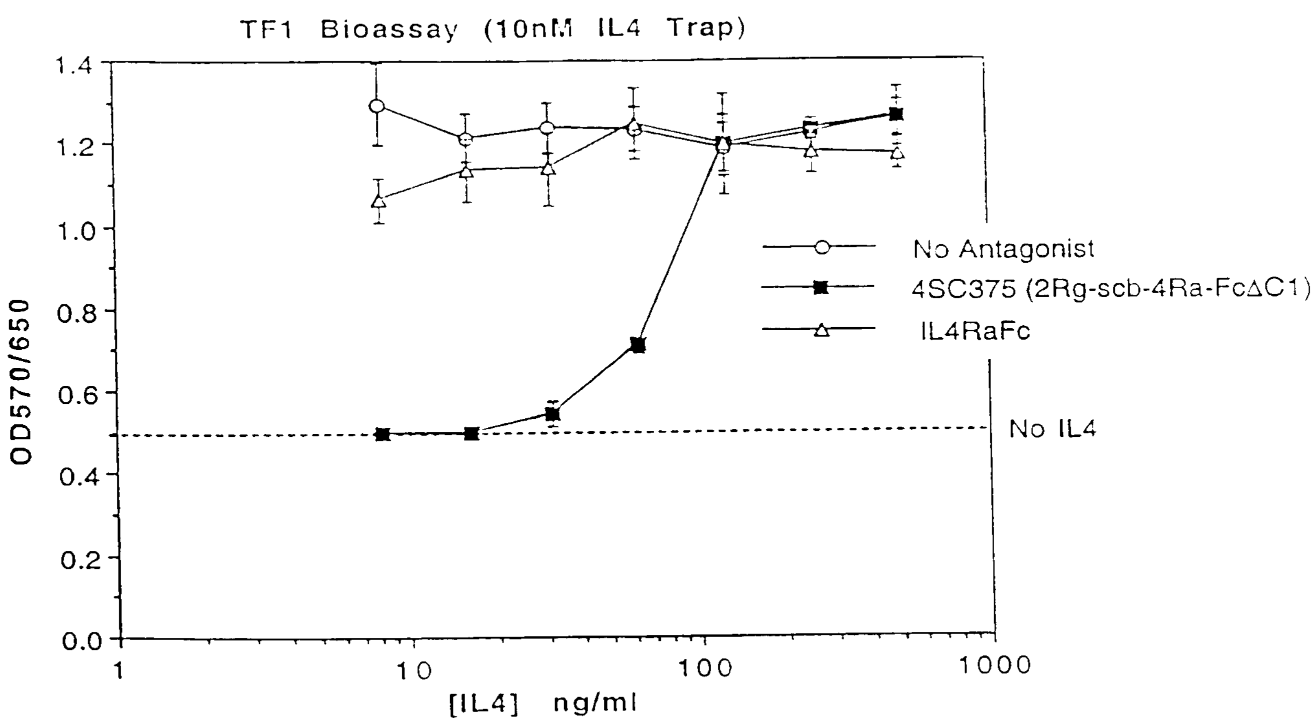
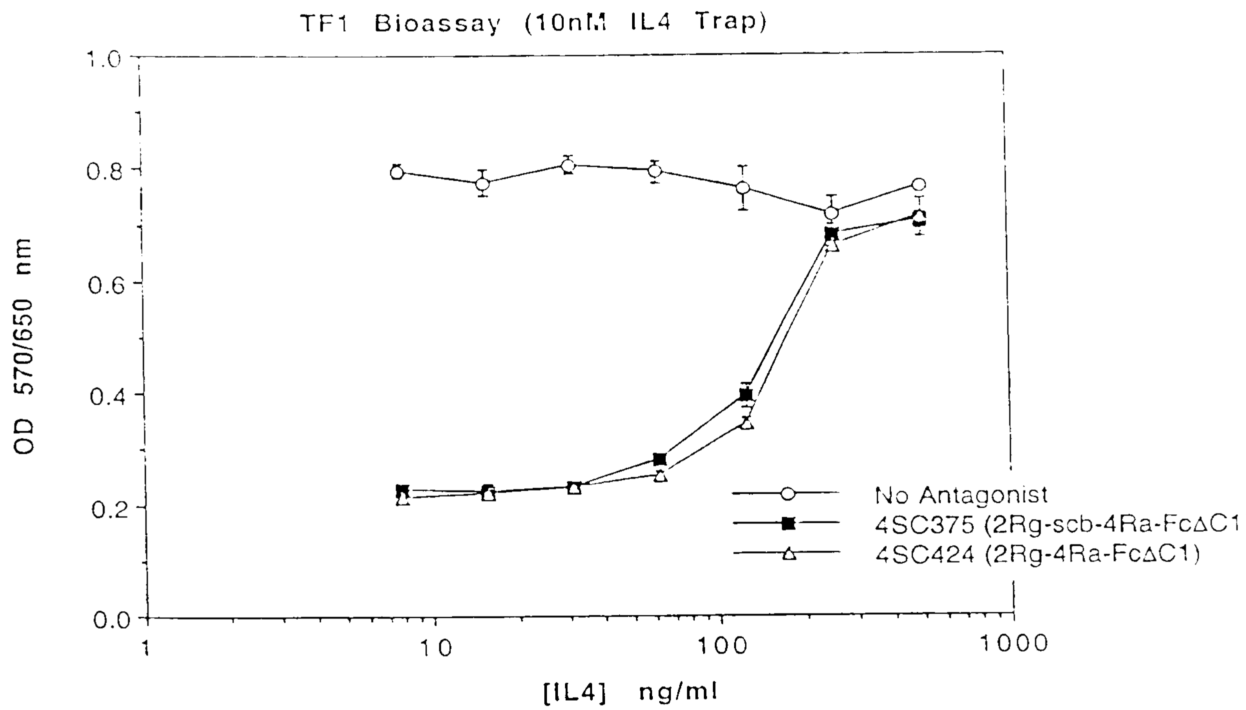

TF1 Bioassay (10nM IL4 Trap)
OD570/650
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
1000
[IL4] ng/ml
No Antagonist
4SC375 (2Rg-scb-4Ra-FcΔC1)
IL4RaFc
No IL4

Figure 28


TF1 Bioassay (10nM IL4 Trap)
OD 570/650 nm
[IL4] ng/ml
No Antagonist
4SC375 (2Rg-scb-4Ra-FcΔC1)
4SC424 (2Rg-4Ra-FcΔC1)

54/74

Figure 29

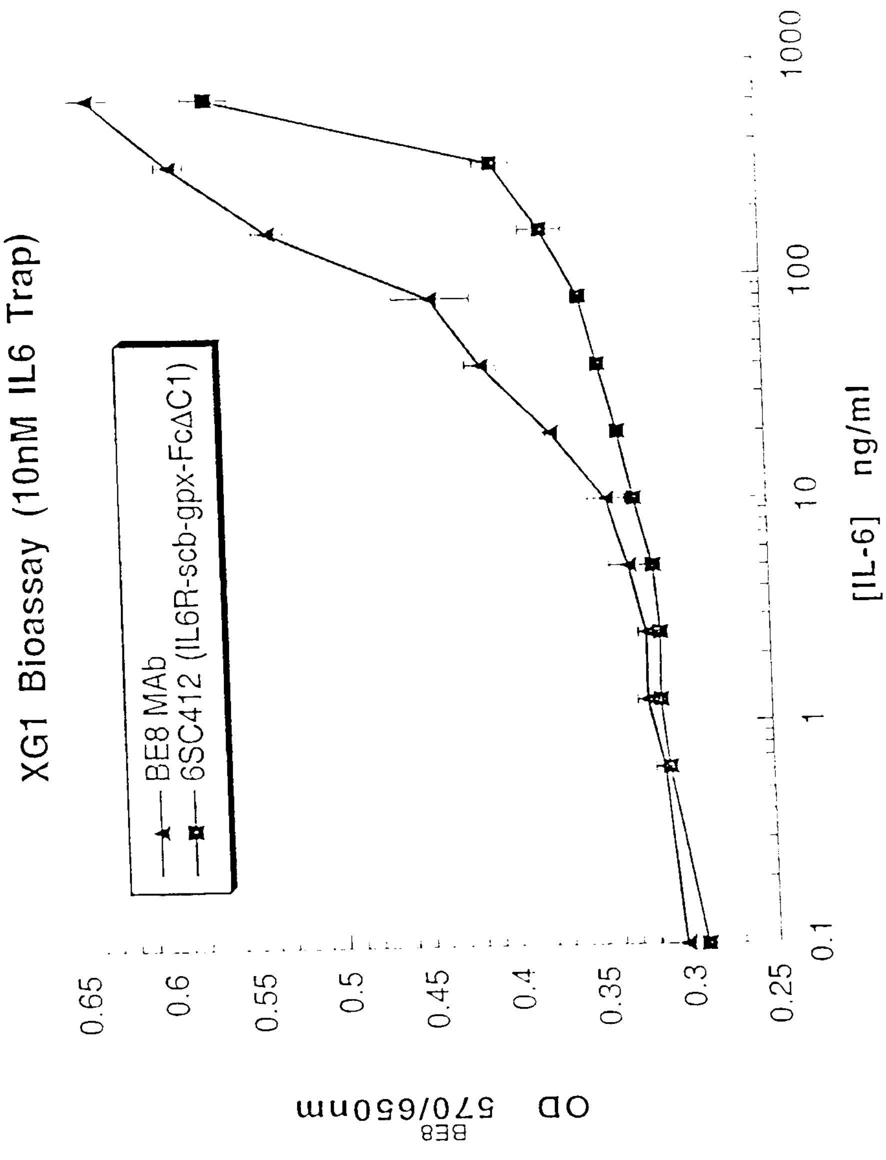


Figure 30

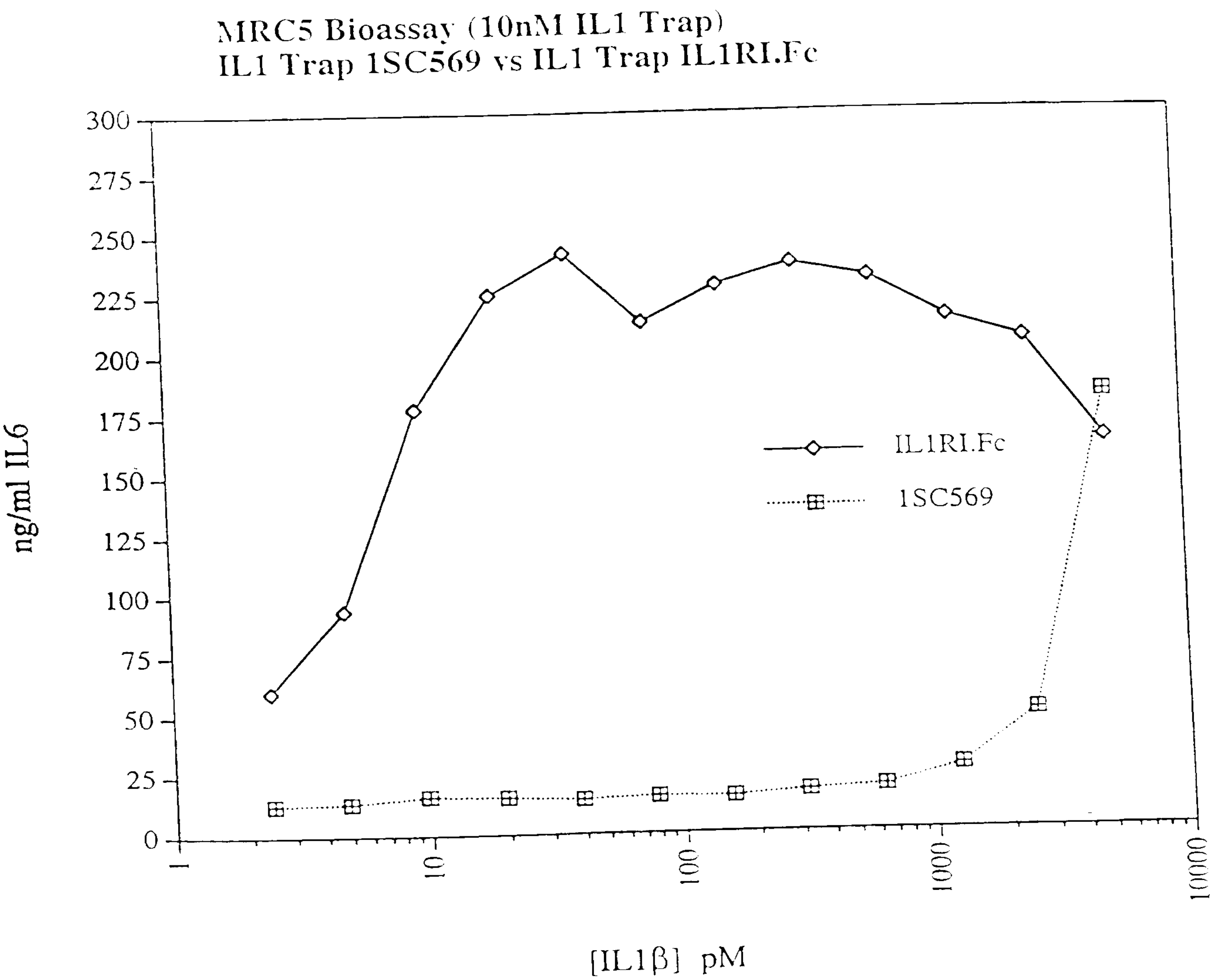

MRC5 Bioassay (10nM IL1 Trap)
IL1 Trap 1SC569 vs IL1 Trap IL1RI.Fc
ng/ml IL6
300
275
250
225
200
175
150
125
100
75
50
25
0
1
10
100
1000
10000
IL1RI.Fc
1SC569
[IL1β] pM

Figure 31A

```
         10         20         30         40
ATG GTG TGG CTT TGC TCT GGG CTC CTG TTC CCT GTG AGC TGC CTG GTC
TAC CAC ACC GAA ACG AGA CCC GAG GAC AAG GGA CAC TCG ACG GAC CAG
Met Val Trp Leu Cys Ser Gly Leu Leu Phe Pro Val Ser Cys Leu Val>

50         60         70         80         90
CTG CTG CAG GTG GCA AGC TCT GGG AAC ATG AAG GTC TTG CAG GAG CCC
GAC GAC GTC CAC CGT TCG AGA CCC TTG TAC TTC CAG AAC GTC CTC GGG
Leu Leu Gln Val Ala Ser Ser Gly Asn Met Lys Val Leu Gln Glu Pro>

 100        110        120        130        140
ACC TGC GTC TCC GAC TAC ATG AGC ATC TCT ACT TGC GAG TGG AAG ATG
TGG ACG CAG AGG CTG ATG TAC TCG TAG AGA TGA ACG CTC ACC TTC TAC
Thr Cys Val Ser Asp Tyr Met Ser Ile Ser Thr Cys Glu Trp Lys Met>

   150        160        170        180        190
AAT GGT CCC ACC AAT TGC AGC ACC GAG CTC CGC CTG TTG TAC CAG CTG
TTA CCA GGG TGG TTA ACG TCG TGG CTC GAG GCG GAC AAC ATG GTC GAC
Asn Gly Pro Thr Asn Cys Ser Thr Glu Leu Arg Leu Leu Tyr Gln Leu>

      200        210        220        230        240
GTT TTT CTG CTC TCC GAA GCC CAC ACG TGT ATC CCT GAG AAC AAC GGA
CAA AAA GAC GAG AGG CTT CGG GTG TGC ACA TAG GGA CTC TTG TTG CCT
Val Phe Leu Leu Ser Glu Ala His Thr Cys Ile Pro Glu Asn Asn Gly>

         250        260        270        280
GGC GCG GGG TGC GTG TGC CAC CTG CTC ATG GAT GAC GTG GTC AGT GCG
CCG CGC CCC ACG CAC ACG GTG GAC GAG TAC CTA CTG CAC CAG TCA CGC
Gly Ala Gly Cys Val Cys His Leu Leu Met Asp Asp Val Val Ser Ala>

290        300        310        320        330
GAT AAC TAT ACA CTG GAC CTG TGG GCT GGG CAG CAG CTG CTG TGG AAG
CTA TTG ATA TGT GAC CTG GAC ACC CGA CCC GTC GTC GAC GAC ACC TTC
Asp Asn Tyr Thr Leu Asp Leu Trp Ala Gly Gln Gln Leu Leu Trp Lys>

  340        350        360        370        380
GGC TCC TTC AAG CCC AGC GAG CAT GTG AAA CCC AGG GCC CCA GGA AAC
CCG AGG AAG TTC GGG TCG CTC GTA CAC TTT GGG TCC CGG GGT CCT TTG
Gly Ser Phe Lys Pro Ser Glu His Val Lys Pro Arg Ala Pro Gly Asn>
```

Figure 31B

```
    390         400         410         420         430
CTG ACA GTT CAC ACC AAT GTT TCC GAC ACT CTG CTG CTG ACC TGG AGC
GAC TGT CAA GTG TGG TTA CAG AGG CTG TGA GAC GAC GAC TGG ACC TCG
Leu Thr Val His Thr Asn Val Ser Asp Thr Leu Leu Leu Thr Trp Ser>

        440         450         460         470         480
AAC CCG TAT CCC CCT GAC AAT TAC CTG TAT AAT CAT CTC ACC TAT GCA
TTG GGC ATA GGG GGA CTG TTA ATG GAC ATA TTA GTA GAG TGG ATA CGT
Asn Pro Tyr Pro Pro Asp Asn Tyr Leu Tyr Asn His Leu Thr Tyr Ala>

            490         500         510         520
GTC AAC ATT TGG AGT GAA AAC GAC CCG GCA GAT TTC AGA ATC TAT AAC
CAG TTG TAA ACC TCA CTT TTG CTG GGC CGT CTA AAG TCT TAG ATA TTG
Val Asn Ile Trp Ser Glu Asn Asp Pro Ala Asp Phe Arg Ile Tyr Asn>

530         540         550         560         570
GTG ACC TAC CTA GAA CCC TCC CTC CGC ATC GCA GCC AGC ACC CTG AAG
CAC TGG ATG GAT CTT GGG AGG GAG GCG TAG CGT CGG TCG TGG GAC TTC
Val Thr Tyr Leu Glu Pro Ser Leu Arg Ile Ala Ala Ser Thr Leu Lys>

  580         590         600         610         620
TCT GGG ATT TCC TAC AGG GCA CGG GTG AGG GCC TGG GCT CAG AGC TAT
AGA CCC TAA AGG ATG TCC CGT GCC CAC TCC CGG ACC CGA GTC TCG ATA
Ser Gly Ile Ser Tyr Arg Ala Arg Val Arg Ala Trp Ala Gln Ser Tyr>

      630         640         650         660         670
AAC ACC ACC TGG AGT GAG TGG AGC CCC AGC ACC AAG TGG CAC AAC TCC
TTG TGG TGG ACC TCA CTC ACC TCG GGG TCG TGG TTC ACC GTG TTG AGG
Asn Thr Thr Trp Ser Glu Trp Ser Pro Ser Thr Lys Trp His Asn Ser>

        680         690         700         710         720
TAC AGG GAG CCC TTC GAG CAG TCC GGT GGG GGC GGG GGC GCC GCG CCT
ATG TCC CTC GGG AAG CTC GTC AGG CCA CCC CCG CCC CCG CGG CGC GGA
Tyr Arg Glu Pro Phe Glu Gln Ser Gly Gly Gly Gly Gly Ala Ala Pro>

            730         740         750         760
ACG GAA ACT CAG CCA CCT GTG ACA AAT TTG AGT GTC TCT GTT GAA AAC
TGC CTT TGA GTC GGT GGA CAC TGT TTA AAC TCA CAG AGA CAA CTT TTG
Thr Glu Thr Gln Pro Pro Val Thr Asn Leu Ser Val Ser Val Glu Asn>
```

Figure 31C

```
770        780        790        800        810
CTC TGC ACA GTA ATA TGG ACA TGG AAT CCA CCC GAG GGA GCC AGC TCA
GAG ACG TGT CAT TAT ACC TGT ACC TTA GGT GGG CTC CCT CGG TCG AGT
Leu Cys Thr Val Ile Trp Thr Trp Asn Pro Pro Glu Gly Ala Ser Ser>

  820        830        840        850        860
AAT TGT AGT CTA TGG TAT TTT AGT CAT TTT GGC GAC AAA CAA GAT AAG
TTA ACA TCA GAT ACC ATA AAA TCA GTA AAA CCG CTG TTT GTT CTA TTC
Asn Cys Ser Leu Trp Tyr Phe Ser His Phe Gly Asp Lys Gln Asp Lys>

    870        880        890        900        910
AAA ATA GCT CCG GAA ACT CGT CGT TCA ATA GAA GTA CCC CTG AAT GAG
TTT TAT CGA GGC CTT TGA GCA GCA AGT TAT CTT CAT GGG GAC TTA CTC
Lys Ile Ala Pro Glu Thr Arg Arg Ser Ile Glu Val Pro Leu Asn Glu>

      920        930        940        950        960
AGG ATT TGT CTG CAA GTG GGG TCC CAG TGT AGC ACC AAT GAG AGT GAG
TCC TAA ACA GAC GTT CAC CCC AGG GTC ACA TCG TGG TTA CTC TCA CTC
Arg Ile Cys Leu Gln Val Gly Ser Gln Cys Ser Thr Asn Glu Ser Glu>

         970        980        990       1000
AAG CCT AGC ATT TTG GTT GAA AAA TGC ATC TCA CCC CCA GAA GGT GAT
TTC GGA TCG TAA AAC CAA CTT TTT ACG TAG AGT GGG GGT CTT CCA CTA
Lys Pro Ser Ile Leu Val Glu Lys Cys Ile Ser Pro Pro Glu Gly Asp>

1010       1020       1030       1040       1050
CCT GAG TCT GCT GTG ACT GAG CTT CAA TGC ATT TGG CAC AAC CTG AGC
GGA CTC AGA CGA CAC TGA CTC GAA GTT ACG TAA ACC GTG TTG GAC TCG
Pro Glu Ser Ala Val Thr Glu Leu Gln Cys Ile Trp His Asn Leu Ser>

 1060       1070       1080       1090       1100
TAC ATG AAG TGT TCT TGG CTC CCT GGA AGG AAT ACC AGT CCC GAC ACT
ATG TAC TTC ACA AGA ACC GAG GGA CCT TCC TTA TGG TCA GGG CTG TGA
Tyr Met Lys Cys Ser Trp Leu Pro Gly Arg Asn Thr Ser Pro Asp Thr>

   1110       1120       1130       1140       1150
AAC TAT ACT CTC TAC TAT TGG CAC AGA AGC CTG GAA AAA ATT CAT CAA
TTG ATA TGA GAG ATG ATA ACC GTG TCT TCG GAC CTT TTT TAA GTA GTT
Asn Tyr Thr Leu Tyr Tyr Trp His Arg Ser Leu Glu Lys Ile His Gln>
```

Figure 31D

```
        1160            1170            1180            1190            1200
  *       *       *       *       *       *       *       *       *       *
TGT GAA AAC ATC TTT AGA GAA GGC CAA TAC TTT GGT TGT TCC TTT GAT
ACA CTT TTG TAG AAA TCT CTT CCG GTT ATG AAA CCA ACA AGG AAA CTA
Cys Glu Asn Ile Phe Arg Glu Gly Gln Tyr Phe Gly Cys Ser Phe Asp>

          1210            1220            1230            1240
      *       *       *       *       *       *       *       *       *
CTG ACC AAA GTG AAG GAT TCC AGT TTT GAA CAA CAC AGT GTC CAA ATA
GAC TGG TTT CAC TTC CTA AGG TCA AAA CTT GTT GTG TCA CAG GTT TAT
Leu Thr Lys Val Lys Asp Ser Ser Phe Glu Gln His Ser Val Gln Ile>

1250            1260            1270            1280            1290
  *       *       *       *       *       *       *       *       *       *
ATG GTC AAG GAT AAT GCA GGA AAA ATT AAA CCA TCC TTC AAT ATA GTG
TAC CAG TTC CTA TTA CGT CCT TTT TAA TTT GGT AGG AAG TTA TAT CAC
Met Val Lys Asp Asn Ala Gly Lys Ile Lys Pro Ser Phe Asn Ile Val>

  1300            1310            1320            1330            1340
      *       *       *       *       *       *       *       *       *
CCT TTA ACT TCC CGT GTG AAA CCT GAT CCT CCA CAT ATT AAA AAC CTC
GGA AAT TGA AGG GCA CAC TTT GGA CTA GGA GGT GTA TAA TTT TTG GAG
Pro Leu Thr Ser Arg Val Lys Pro Asp Pro Pro His Ile Lys Asn Leu>

    1350            1360            1370            1380            1390
  *       *       *       *       *       *       *       *       *       *
TCC TTC CAC AAT GAT GAC CTA TAT GTG CAA TGG GAG AAT CCA CAG AAT
AGG AAG GTG TTA CTA CTG GAT ATA CAC GTT ACC CTC TTA GGT GTC TTA
Ser Phe His Asn Asp Asp Leu Tyr Val Gln Trp Glu Asn Pro Gln Asn>

        1400            1410            1420            1430            1440
  *       *       *       *       *       *       *       *       *       *
TTT ATT AGC AGA TGC CTA TTT TAT GAA GTA GAA GTC AAT AAC AGC CAA
AAA TAA TCG TCT ACG GAT AAA ATA CTT CAT CTT CAG TTA TTG TCG GTT
Phe Ile Ser Arg Cys Leu Phe Tyr Glu Val Glu Val Asn Asn Ser Gln>

            1450            1460            1470            1480
      *       *       *       *       *       *       *       *       *
ACT GAG ACA CAT AAT GTT TTC TAC GTC CAA GAG GCT AAA TGT GAG AAT
TGA CTC TGT GTA TTA CAA AAG ATG CAG GTT CTC CGA TTT ACA CTC TTA
Thr Glu Thr His Asn Val Phe Tyr Val Gln Glu Ala Lys Cys Glu Asn>

1490            1500            1510            1520            1530
  *       *       *       *       *       *       *       *       *       *
CCA GAA TTT GAG AGA AAT GTG GAG AAT ACA TCT TGT TTC ATG GTC CCT
GGT CTT AAA CTC TCT TTA CAC CTC TTA TGT AGA ACA AAG TAC CAG GGA
Pro Glu Phe Glu Arg Asn Val Glu Asn Thr Ser Cys Phe Met Val Pro>
```

Figure 31E

```
  1540      1550      1560      1570      1580
GGT GTT CTT CCT GAT ACT TTG AAC ACA GTC AGA ATA AGA GTC AAA ACA
CCA CAA GAA GGA CTA TGA AAC TTG TGT CAG TCT TAT TCT CAG TTT TGT
Gly Val Leu Pro Asp Thr Leu Asn Thr Val Arg Ile Arg Val Lys Thr>

   1590      1600      1610      1620      1630
AAT AAG TTA TGC TAT GAG GAT GAC AAA CTC TGG AGT AAT TGG AGC CAA
TTA TTC AAT ACG ATA CTC CTA CTG TTT GAG ACC TCA TTA ACC TCG GTT
Asn Lys Leu Cys Tyr Glu Asp Asp Lys Leu Trp Ser Asn Trp Ser Gln>

     1640      1650      1660      1670      1680
GAA ATG AGT ATA GGT AAG AAG CGC AAT TCC ACA ACC GGA GAC AAA ACT
CTT TAC TCA TAT CCA TTC TTC GCG TTA AGG TGT TGG CCT CTG TTT TGA
Glu Met Ser Ile Gly Lys Lys Arg Asn Ser Thr Thr Gly Asp Lys Thr>

       1690      1700      1710      1720
CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA
GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT
His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser>

1730      1740      1750      1760      1770
GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG
CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg>

  1780      1790      1800      1810      1820
ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT
TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro>

    1830      1840      1850      1860      1870
GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC
CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG
Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala>

      1880      1890      1900      1910      1920
AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC
TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG
Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val>
```

Figure 31F

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      1930        1940        1950        1960
AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC
TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG
Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr>

1970        1980        1990        2000        2010
AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC
TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG
Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>

 2020        2030        2040        2050        2060
ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG
TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu>

   2070        2080        2090        2100        2110
CCC CCA TCC CGG GAG GAG ATG ACC AAG AAC CAG GTC AGC CTG ACC TGC
GGG GGT AGG GCC CTC CTC TAC TGG TTC TTG GTC CAG TCG GAC TGG ACG
Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys>

     2120        2130        2140        2150        2160
CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC
GAC CAG TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser>

       2170        2180        2190        2200
AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC
TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp>

2210        2220        2230        2240        2250
TCC GAC GGC TCC TTC TTC CTC TAT AGC AAG CTC ACC GTG GAC AAG AGC
AGG CTG CCG AGG AAG AAG GAG ATA TCG TTC GAG TGG CAC CTG TTC TCG
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser>

 2260        2270        2280        2290        2300
AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT
TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala>
```

Figure 31G

```
   2310        2320        2330        2340        2350
 .   .   .   .   .   .   .   .   .   .
CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA
GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT
Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys>

  .
TGA
ACT
***>
```

Figure 32A

```
                10                  20                  30                  40
        *        *        *         *         *        *         *        *        *
ATG GTG TGG CCG GCG CGG CTC TGC GGG CTG TGG GCG CTG CTG CTC TGC
TAC CAC ACC GGC CGC GCC GAG ACG CCC GAC ACC CGC GAC GAC GAG ACG
Met Val Trp Pro Ala Arg Leu Cys Gly Leu Trp Ala Leu Leu Leu Cys>

50                  60                  70                  80                  90
 *        *        *         *         *        *         *        *        *        *
GCC GGC GGC GGG GGC GGG GGC GGG GGC GCC GCG CCT ACG GAA ACT CAG
CGG CCG CCG CCC CCG CCC CCG CCC CCG CGG CGC GGA TGC CTT TGA GTC
Ala Gly Gly Gly Gly Gly Gly Gly Gly Ala Ala Pro Thr Glu Thr Gln>

   100                 110                 120                 130                 140
    *        *        *         *         *        *         *        *        *
CCA CCT GTG ACA AAT TTG AGT GTC TCT GTT GAA AAC CTC TGC ACA GTA
GGT GGA CAC TGT TTA AAC TCA CAG AGA CAA CTT TTG GAG ACG TGT CAT
Pro Pro Val Thr Asn Leu Ser Val Ser Val Glu Asn Leu Cys Thr Val>

       150                 160                 170                 180                 190
 *        *        *         *         *        *         *        *        *        *
ATA TGG ACA TGG AAT CCA CCC GAG GGA GCC AGC TCA AAT TGT AGT CTA
TAT ACC TGT ACC TTA GGT GGG CTC CCT CGG TCG AGT TTA ACA TCA GAT
Ile Trp Thr Trp Asn Pro Pro Glu Gly Ala Ser Ser Asn Cys Ser Leu>

          200                 210                 220                 230                 240
   *        *        *         *         *        *         *        *        *        *
TGG TAT TTT AGT CAT TTT GGC GAC AAA CAA GAT AAG AAA ATA GCT CCG
ACC ATA AAA TCA GTA AAA CCG CTG TTT GTT CTA TTC TTT TAT CGA GGC
Trp Tyr Phe Ser His Phe Gly Asp Lys Gln Asp Lys Lys Ile Ala Pro>

                 250                 260                 270                 280
        *        *        *         *         *        *         *        *        *
GAA ACT CGT CGT TCA ATA GAA GTA CCC CTG AAT GAG AGG ATT TGT CTG
CTT TGA GCA GCA AGT TAT CTT CAT GGG GAC TTA CTC TCC TAA ACA GAC
Glu Thr Arg Arg Ser Ile Glu Val Pro Leu Asn Glu Arg Ile Cys Leu>

290                 300                 310                 320                 330
 *        *        *         *         *        *         *        *        *        *
CAA GTG GGG TCC CAG TGT AGC ACC AAT GAG AGT GAG AAG CCT AGC ATT
GTT CAC CCC AGG GTC ACA TCG TGG TTA CTC TCA CTC TTC GGA TCG TAA
Gln Val Gly Ser Gln Cys Ser Thr Asn Glu Ser Glu Lys Pro Ser Ile>

   340                 350                 360                 370                 380
    *        *        *         *         *        *         *        *        *
TTG GTT GAA AAA TGC ATC TCA CCC CCA GAA GGT GAT CCT GAG TCT GCT
AAC CAA CTT TTT ACG TAG AGT GGG GGT CTT CCA CTA GGA CTC AGA CGA
Leu Val Glu Lys Cys Ile Ser Pro Pro Glu Gly Asp Pro Glu Ser Ala>
```

Figure 32B

```
    390         400         410         420         430
 *   *     *     *     *     *     *     *     *     *
GTG ACT GAG CTT CAA TGC ATT TGG CAC AAC CTG AGC TAC ATG AAG TGT
CAC TGA CTC GAA GTT ACG TAA ACC GTG TTG GAC TCG ATG TAC TTC ACA
Val Thr Glu Leu Gln Cys Ile Trp His Asn Leu Ser Tyr Met Lys Cys>

        440         450         460         470         480
  *     *     *     *     *     *     *     *     *     *
TCT TGG CTC CCT GGA AGG AAT ACC AGT CCC GAC ACT AAC TAT ACT CTC
AGA ACC GAG GGA CCT TCC TTA TGG TCA GGG CTG TGA TTG ATA TGA GAG
Ser Trp Leu Pro Gly Arg Asn Thr Ser Pro Asp Thr Asn Tyr Thr Leu>

            490         500         510         520
      *     *     *     *     *     *     *     *     *
TAC TAT TGG CAC AGA AGC CTG GAA AAA ATT CAT CAA TGT GAA AAC ATC
ATG ATA ACC GTG TCT TCG GAC CTT TTT TAA GTA GTT ACA CTT TTG TAG
Tyr Tyr Trp His Arg Ser Leu Glu Lys Ile His Gln Cys Glu Asn Ile>

530         540         550         560         570
 *     *     *     *     *     *     *     *     *     *
TTT AGA GAA GGC CAA TAC TTT GGT TGT TCC TTT GAT CTG ACC AAA GTG
AAA TCT CTT CCG GTT ATG AAA CCA ACA AGG AAA CTA GAC TGG TTT CAC
Phe Arg Glu Gly Gln Tyr Phe Gly Cys Ser Phe Asp Leu Thr Lys Val>

   580         590         600         610         620
     *     *     *     *     *     *     *     *     *
AAG GAT TCC AGT TTT GAA CAA CAC AGT GTC CAA ATA ATG GTC AAG GAT
TTC CTA AGG TCA AAA CTT GTT GTG TCA CAG GTT TAT TAC CAG TTC CTA
Lys Asp Ser Ser Phe Glu Gln His Ser Val Gln Ile Met Val Lys Asp>

     630         640         650         660         670
 *     *     *     *     *     *     *     *     *     *
AAT GCA GGA AAA ATT AAA CCA TCC TTC AAT ATA GTG CCT TTA ACT TCC
TTA CGT CCT TTT TAA TTT GGT AGG AAG TTA TAT CAC GGA AAT TGA AGG
Asn Ala Gly Lys Ile Lys Pro Ser Phe Asn Ile Val Pro Leu Thr Ser>

        680         690         700         710         720
  *     *     *     *     *     *     *     *     *     *
CGT GTG AAA CCT GAT CCT CCA CAT ATT AAA AAC CTC TCC TTC CAC AAT
GCA CAC TTT GGA CTA GGA GGT GTA TAA TTT TTG GAG AGG AAG GTG TTA
Arg Val Lys Pro Asp Pro Pro His Ile Lys Asn Leu Ser Phe His Asn>

            730         740         750         760
      *     *     *     *     *     *     *     *     *
GAT GAC CTA TAT GTG CAA TGG GAG AAT CCA CAG AAT TTT ATT AGC AGA
CTA CTG GAT ATA CAC GTT ACC CTC TTA GGT GTC TTA AAA TAA TCG TCT
Asp Asp Leu Tyr Val Gln Trp Glu Asn Pro Gln Asn Phe Ile Ser Arg>
```

Figure 32C

```
770       780       790       800       810
TGC CTA TTT TAT GAA GTA GAA GTC AAT AAC AGT CAA ACT GAG ACA CAT
ACG GAT AAA ATA CTT CAT CTT CAG TTA TTG TCG GTT TGA CTC TGT GTA
Cys Leu Phe Tyr Glu Val Glu Val Asn Asn Ser Gln Thr Glu Thr His>

820       830       840       850       860
AAT GTT TTC TAC GTC CAA GAG GCT AAA TGT GAG AAT CCA GAA TTT GAG
TTA CAA AAG ATG CAG GTT CTC CGA TTT ACA CTC TTA GGT CTT AAA CTC
Asn Val Phe Tyr Val Gln Glu Ala Lys Cys Glu Asn Pro Glu Phe Glu>

870       880       890       900       910
AGA AAT GTG GAG AAT ACA TCT TGT TTC ATG GTC CCT GGT GTT CTT CCT
TCT TTA CAC CTC TTA TGT AGA ACA AAG TAC CAG GGA CCA CAA GAA GGA
Arg Asn Val Glu Asn Thr Ser Cys Phe Met Val Pro Gly Val Leu Pro>

920       930       940       950       960
GAT ACT TTG AAC ACA GTC AGA ATA AGA GTC AAA ACA AAT AAG TTA TGC
CTA TGA AAC TTG TGT CAG TCT TAT TCT CAG TTT TGT TTA TTC AAT ACG
Asp Thr Leu Asn Thr Val Arg Ile Arg Val Lys Thr Asn Lys Leu Cys>

970       980       990       1000
TAT GAG GAT GAC AAA CTC TGG AGT AAT TGG AGC CAA GAA ATG AGT ATA
ATA CTC CTA CTG TTT GAG ACC TCA TTA ACC TCG GTT CTT TAC TCA TAT
Tyr Glu Asp Asp Lys Leu Trp Ser Asn Trp Ser Gln Glu Met Ser Ile>

1010      1020      1030      1040      1050
GGT AAG AAG CGC AAT TCC ACA GGC GCG CCT AGT GGT GGA GGT GGC CGG
CCA TTC TTC GCG TTA AGG TGT CCG CGC GGA TCA CCA CCT CCA CCG GCC
Gly Lys Lys Arg Asn Ser Thr Gly Ala Pro Ser Gly Gly Gly Gly Arg>

1060      1070      1080      1090      1100
CCC GCA AGC TCT GGG AAC ATG AAG GTC TTG CAG GAG CCC ACC TGC GTC
GGG CGT TCG AGA CCC TTG TAC TTC CAG AAC GTC CTC GGG TGG ACG CAG
Pro Ala Ser Ser Gly Asn Met Lys Val Leu Gln Glu Pro Thr Cys Val>

1110      1120      1130      1140      1150
TCC GAC TAC ATG AGC ATC TCT ACT TGC GAG TGG AAG ATG AAT GGT CCC
AGG CTG ATG TAC TCG TAG AGA TGA ACG CTC ACC TTC TAC TTA CCA GGG
Ser Asp Tyr Met Ser Ile Ser Thr Cys Glu Trp Lys Met Asn Gly Pro>
```

Figure 32D

```
        1160        1170          1180          1190          1200
  *      *      *      *      *      *      *      *      *      *
ACC AAT TGC AGC ACC GAG CTC CGC CTG TTG TAC CAG CTG GTT TTT CTG
TGG TTA ACG TCG TGG CTC GAG GCG GAC AAC ATG GTC GAC CAA AAA GAC
Thr Asn Cys Ser Thr Glu Leu Arg Leu Leu Tyr Gln Leu Val Phe Leu>

           1210          1220          1230          1240
      *      *      *      *      *      *      *      *      *
CTC TCC GAA GCC CAC ACG TGT ATC CCT GAG AAC AAC GGA GGC GCG GGG
GAG AGG CTT CGG GTG TGC ACA TAG GGA CTC TTG TTG CCT CCG CGC CCC
Leu Ser Glu Ala His Thr Cys Ile Pro Glu Asn Asn Gly Gly Ala Gly>

1250        1260          1270          1280          1290
  *      *      *      *      *      *      *      *      *      *
TGC GTG TGC CAC CTG CTC ATG GAT GAC GTG GTC AGT GCG GAT AAC TAT
ACG CAC ACG GTG GAC GAG TAC CTA CTG CAC CAG TCA CGC CTA TTG ATA
Cys Val Cys His Leu Leu Met Asp Asp Val Val Ser Ala Asp Asn Tyr>

 1300         1310          1320          1330          1340
      *      *      *      *      *      *      *      *      *
ACA CTG GAC CTG TGG GCT GGG CAG CAG CTG CTG TGG AAG GGC TCC TTC
TGT GAC CTG GAC ACC CGA CCC GTC GTC GAC GAC ACC TTC CCG AGG AAG
Thr Leu Asp Leu Trp Ala Gly Gln Gln Leu Leu Trp Lys Gly Ser Phe>

   1350          1360          1370          1380          1390
  *      *      *      *      *      *      *      *      *      *
AAG CCC AGC GAG CAT GTG AAA CCC AGG GCC CCA GGA AAC CTG ACA GTT
TTC GGG TCG CTC GTA CAC TTT GGG TCC CGG GGT CCT TTG GAC TGT CAA
Lys Pro Ser Glu His Val Lys Pro Arg Ala Pro Gly Asn Leu Thr Val>

       1400          1410          1420          1430          1440
  *      *      *      *      *      *      *      *      *      *
CAC ACC AAT GTC TCC GAC ACT CTG CTG CTG ACC TGG AGC AAC CCG TAT
GTG TGG TTA CAG AGG CTG TGA GAC GAC GAC TGG ACC TCG TTG GGC ATA
His Thr Asn Val Ser Asp Thr Leu Leu Leu Thr Trp Ser Asn Pro Tyr>

          1450          1460          1470          1480
      *      *      *      *      *      *      *      *      *
CCC CCT GAC AAT TAC CTG TAT AAT CAT CTC ACC TAT GCA GTC AAC ATT
GGG GGA CTG TTA ATG GAC ATA TTA GTA GAG TGG ATA CGT CAG TTG TAA
Pro Pro Asp Asn Tyr Leu Tyr Asn His Leu Thr Tyr Ala Val Asn Ile>

1490        1500          1510          1520          1530
  *      *      *      *      *      *      *      *      *      *
TGG AGT GAA AAC GAC CCG GCA GAT TTC AGA ATC TAT AAC GTG ACC TAC
ACC TCA CTT TTG CTG GGC CGT CTA AAG TCT TAG ATA TTG CAC TGG ATG
Trp Ser Glu Asn Asp Pro Ala Asp Phe Arg Ile Tyr Asn Val Thr Tyr>
```

Figure 32E

```
1540        1550        1560        1570        1580
CTA GAA CCC TCC CTC CGC ATC GCA GCC AGC ACC CTG AAG TCT GGG ATT
GAT CTT GGG AGG GAG GCG TAG CGT CGG TCG TGG GAC TTC AGA CCC TAA
Leu Glu Pro Ser Leu Arg Ile Ala Ala Ser Thr Leu Lys Ser Gly Ile>

  1590        1600        1610        1620        1630
TCC TAC AGG GCA CGG GTG AGG GCC TGG GCT CAG TGC TAT AAC ACC ACC
AGG ATG TCC CGT GCC CAC TCC CGG ACC CGA GTC ACG ATA TTG TGG TGG
Ser Tyr Arg Ala Arg Val Arg Ala Trp Ala Gln Cys Tyr Asn Thr Thr>

     1640        1650        1660        1670        1680
TGG AGT GAG TGG AGC CCC AGC ACC AAG TGG CAC AAC TCC TAC AGG GAG
ACC TCA CTC ACC TCG GGG TCG TGG TTC ACC GTG TTG AGG ATG TCC CTC
Trp Ser Glu Trp Ser Pro Ser Thr Lys Trp His Asn Ser Tyr Arg Glu>

        1690        1700        1710        1720
CCC TTC GAG CAG TCC GGA GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA
GGG AAG CTC GTC AGG CCT CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT
Pro Phe Glu Gln Ser Gly Asp Lys Thr His Thr Cys Pro Pro Cys Pro>

1730        1740        1750        1760        1770
GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA
CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT
Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys>

 1780        1790        1800        1810        1820
CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG
GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC
Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val>

   1830        1840        1850        1860        1870
GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC
CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG
Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr>

      1880        1890        1900        1910        1920
GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG
CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC
Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu>
```

Figure 32F

```
        1930        1940        1950        1960
CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC
GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG
Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His>

1970        1980        1990        2000        2010
CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA
GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT
Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys>

 2020        2030        2040        2050        2060
GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG
CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC
Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln>

    2070        2080        2090        2100        2110
CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAG GAG ATG
GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTC CTC TAC
Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met>

       2120        2130        2140        2150        2160
ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC
TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG
Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro>

          2170        2180        2190        2200
AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC
TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG
Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn>

2210        2220        2230        2240        2250
TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC
ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG
Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu>

 2260        2270        2280        2290        2300
TAT AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC
ATA TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG
Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val>
```

Figure 32G

```
   2310          2320          2330          2340          2350
*     *     *     *     *     *     *     *     *     *
TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG
AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC
Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln>

      2360          2370          2380
  *     *     *     *     *     *
AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT
Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
```

Figure 33

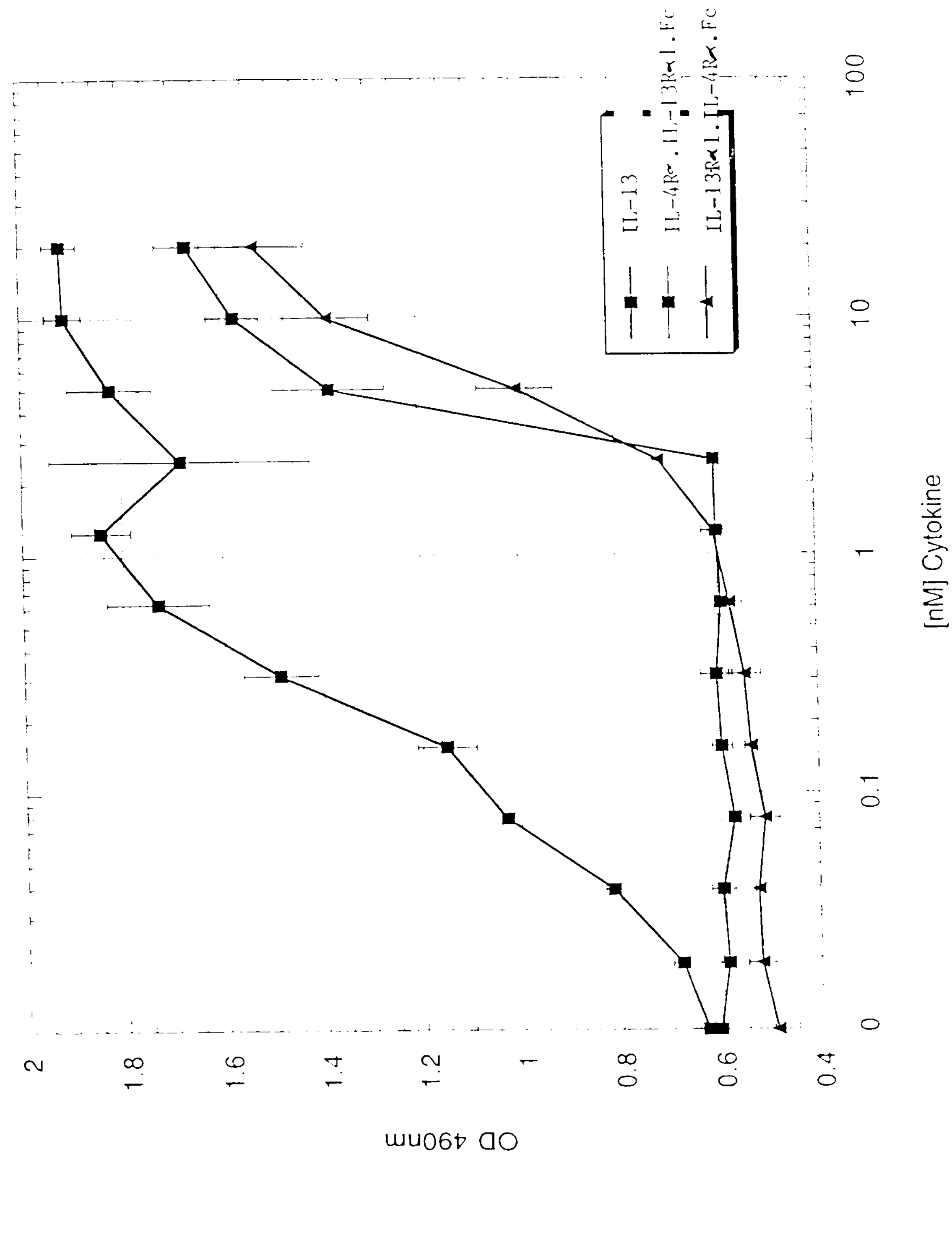

OD 490nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0
0.1
1
10
100
[nM] Cytokine
IL-13
IL-4Rα.IL-13Rα1.Fc
IL-13Rα1.IL-4Rα.Fc

Figure 34

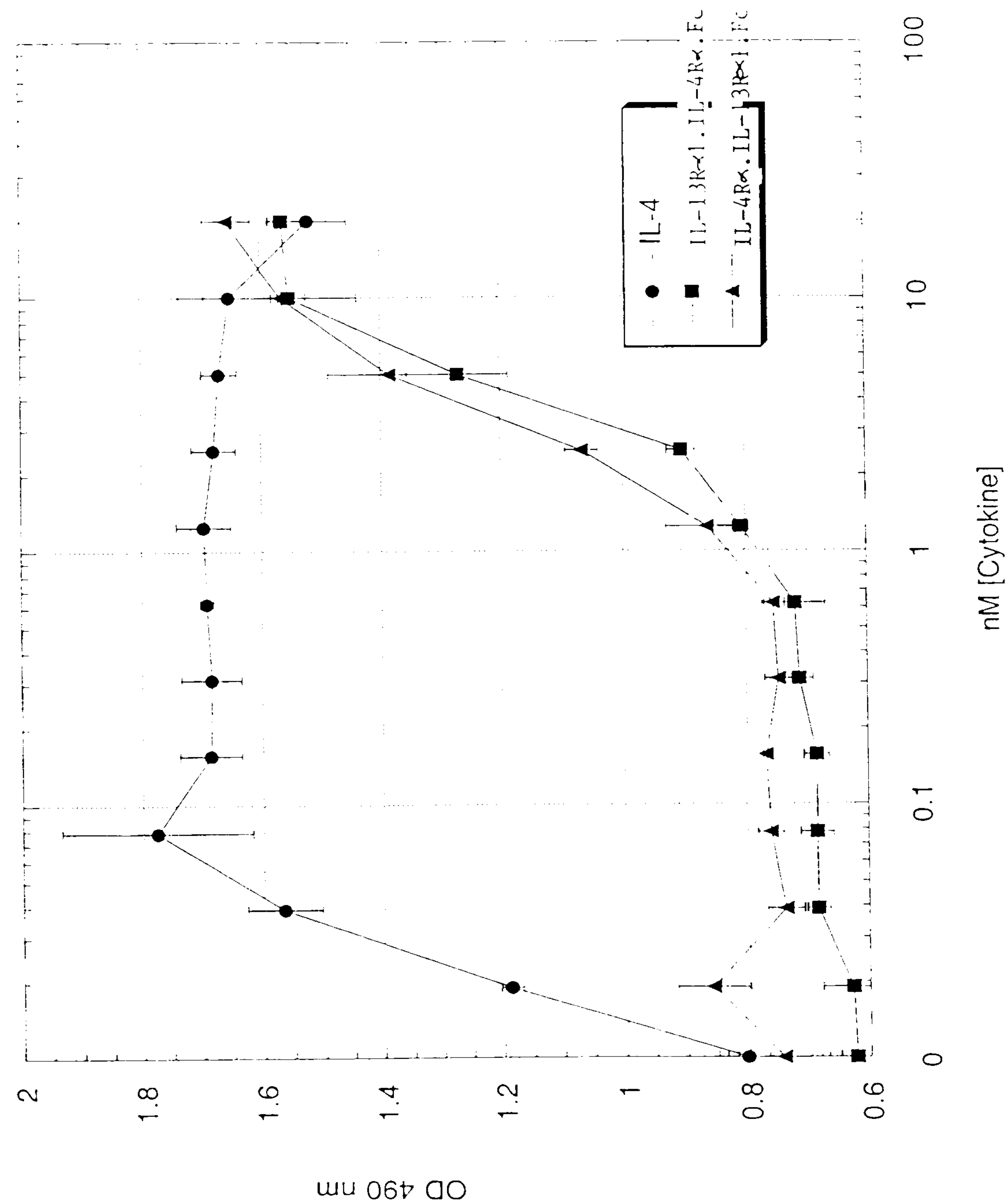

IL-4
IL-13Rα1.IL-4Rα.Fc
IL-4Rα.IL-13Rα1.Fc
OD 490 nm
nM [Cytokine]
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0
0.1
1
10
100

Figure 35

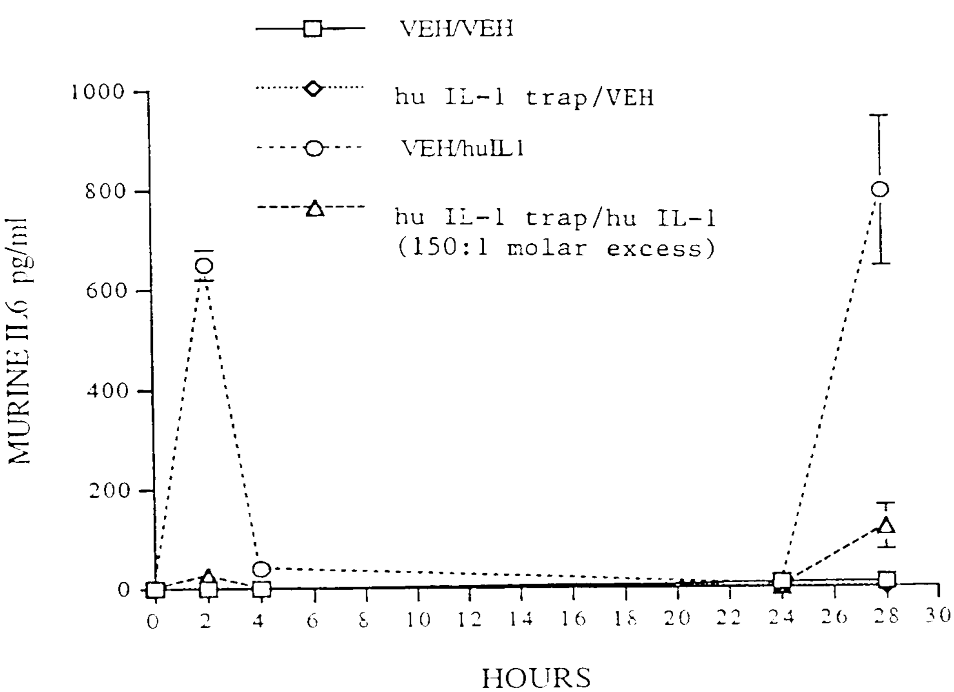
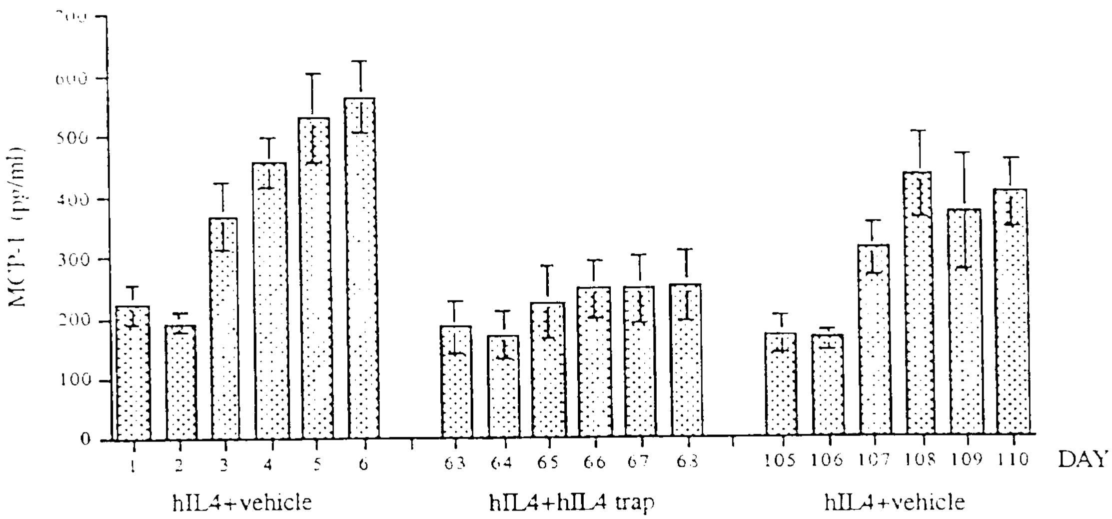
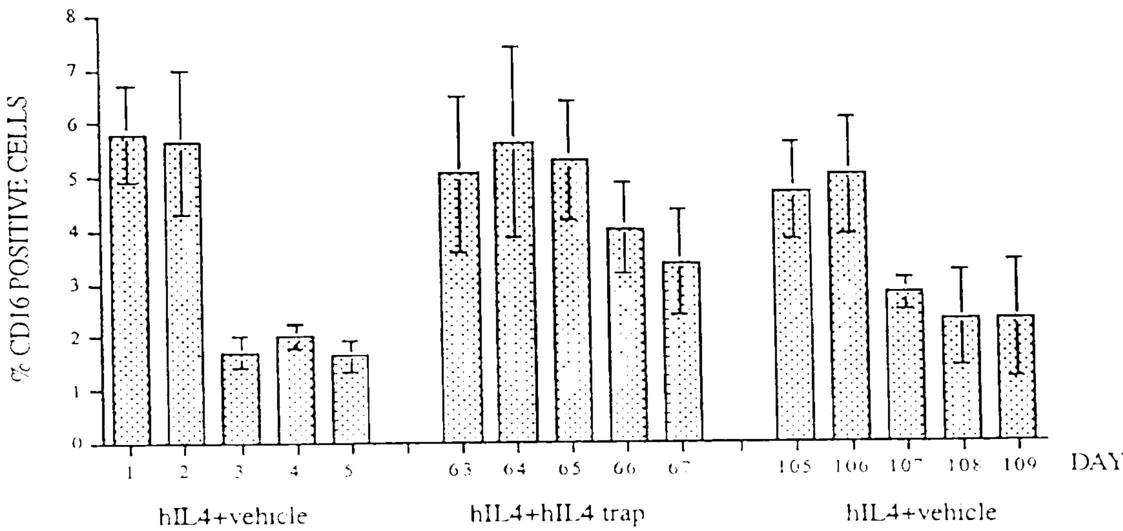

VEH/VEH
hu IL-1 trap/VEH
VEH/huIL1
hu IL-1 trap/hu IL-1
(150:1 molar excess)
1000
800
600
400
200
0
MURINE IL6 pg/ml
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
HOURS

Figure 36A



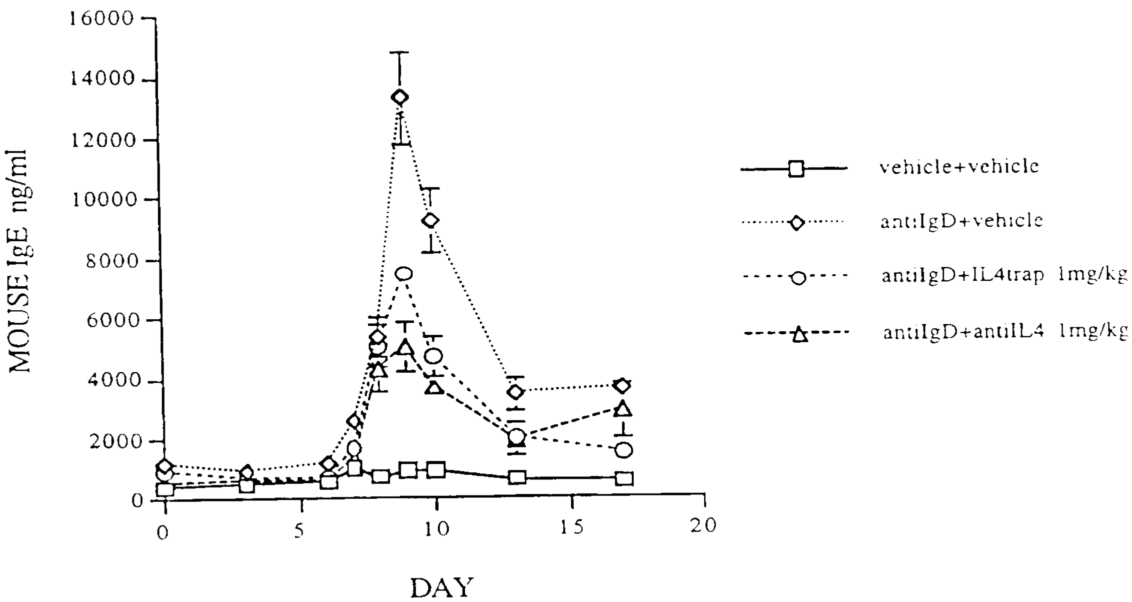
Part 1 Part 2 Part 3

Figure 36B



Part 1 Part 2 Part 3

Figure 37


16000
14000
12000
10000
8000
6000
4000
2000
0
MOUSE IgE ng/ml
0
5
10
15
20
DAY
vehicle+vehicle
antiIgD+vehicle
antiIgD+IL4trap 1mg/kg
antiIgD+antiIL4 1mg/kg

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/22253

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 H04L29/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 H04L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BLOBEL B ET AL: "CORBA security services for health information systems" INTERNATIONAL JOURNAL OF MEDICAL INFORMATICS,IR,ELSEVIER SCIENTIFIC PUBLISHERS, SHANNON, vol. 52, no. 1-3, 1 October 1998 (1998-10-01), pages 29-37, XP004153668 ISSN: 1386-5056 abstract page 33, right-hand column, line 22 -page 35, right-hand column, line 37	1-22

-/--

[X] Further documents are listed in the continuation of box C.

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* Special categories of cited documents :

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2000	13/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Adkhis, F

1

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/22253

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KATSIKAS S K D ET AL: "Using trusted third parties for secure telemedical applications over the WWW: The EUROMED-ETS approach" INTERNATIONAL JOURNAL OF MEDICAL INFORMATICS, IR, ELSEVIER SCIENTIFIC PUBLISHERS, SHANNON, vol. 49, no. 1, pages 59-68, XP004149462 ISSN: 1386-5056 abstract page 60, left-hand column, line 37 -right-hand column, line 32 page 64, right-hand column, line 26 -page 65, left-hand column, line 30 page 67, left-hand column, line 40 -page 68, left-hand column, line 25	1-22

1

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(11) International Publication Number: WO 00/18932

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(30) Priority Data:
60/101,858 25 September 1998 (25.09.98) US
09/313,942 19 May 1999 (19.05.99) US

(71) Applicant *(for all designated States except US)*: REGENERON PHARMACEUTICALS, INC. [US/US]; 777 Old Saw Mill River Road, Tarrytown, NY 10591-6707 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: STAHL, Neil [US/US]; RD #10, Kent Shore Drive, Carmel, NY 10512 (US). YANCOPOULOS, George, D. [US/US]; 1519 Baptist Church Road, Yorktown Heights, NY 10598 (US).

(74) Agents: SORRENTINO, Joseph, M.; Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US) et al.

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report: 2 November 2000 (02.11.00)

(54) Title: RECEPTOR BASED ANTAGONISTS AND METHODS OF MAKING AND USING

(57) Abstract

The present invention provides a fusion polypeptide capable of binding a cytokine to form a nonfunctional complex. It also provides a nucleic acid sequence encoding the fusion polypeptide and methods of making and uses for the fusion polypeptide.

*(Referred to in PCT Gazette No. 35/2000, Section II)

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INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT S 99/22045

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/62 C07K14/715 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 23881 A (CELL GENESYS INC) 8 August 1996 (1996-08-08) page 16, line 14 -page 19, line 25 page 47, line 5 -page 48, line 13 page 51, line 12 -page 52, line 3 page 54, line 31 -page 55, line 17 page 58, line 10 -page 59, line 3 page 61, line 33 -page 62, line 20 page 65, line 10 -page 66, line 3 page 69, line 5 - line 25 page 72, line 4 - line 22 page 75, line 10 -page 76, line 3; claims 1,3,4,6,11,12,14,15,17,23,25,26,ETC..; figure 5 ---	1-4,9-25

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[X] Further documents are listed in the continuation of box C.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 July 2000	20.07.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

3

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC S 99/22045

C.(Continuation) DOCUMENTS CONSIDERED E RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 22914 A (HUTCHINSON FRED CANCER RES ;GREENBERG PHILIP D (US); NELSON BRAD H) 13 October 1994 (1994-10-13) the whole document	1-4,9-25
X	WO 96 11213 A (AMGEN BOULDER INC) 18 April 1996 (1996-04-18) cited in the application the whole document	1-4,9-25
X	WO 97 15669 A (US HEALTH) 1 May 1997 (1997-05-01) page 18, line 27 - line 34; claims 12,16,28	1-4, 10-25
X	WO 93 19163 A (YEDA RES & DEV) 30 September 1993 (1993-09-30) abstract page 7, line 8 -page 8, line 1 page 8, line 31 - line 36 page 17, line 21 - line 24; claims 1,12,16	1,2,4, 10-25
X	WO 95 06737 A (PRENDERGAST KENNETH FRANCIS) 9 March 1995 (1995-03-09) see seq.ID.16 and the claims	1-4, 10-25
X	WO 93 19777 A (IMMUNEX CORP) 14 October 1993 (1993-10-14) claims examples 12,13	1-4, 10-25
X	EP 0 835 939 A (HOECHST AG ;GEN HOSPITAL CORP (US)) 15 April 1998 (1998-04-15) claims 8,9,17,21; example 2	1,2,4, 9-25
X	WO 97 31946 A (GENETICS INST) 4 September 1997 (1997-09-04) claims 11,27,28; example 4	1,2,4, 10-25
X	EP 0 533 006 A (HOFFMANN LA ROCHE) 24 March 1993 (1993-03-24) claims 1,3,5,8-10; examples 12,13	1,2,4, 10-25
X	WO 96 35783 A (DARTMOUTH COLLEGE ;CHIRON CORP (US)) 14 November 1996 (1996-11-14) claims 4,5	1,2,4, 10-25

-/--

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SEIPELT I ET AL: "Overexpression, purification, and use of a soluble human interleukin-4 receptor alpha-chain/Ig gamma 1 fusion protein for ligand binding studies. Characterization of ligand binding to soluble IL-4 receptor alpha-chain by surface plasmon resonance measurements and by microtiter-plate-based ELISA with" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS,US,ACADEMIC PRESS INC. ORLANDO, FL, vol. 239, 20 October 1997 (1997-10-20), pages 534-542, XP002105430 ISSN: 0006-291X the whole document ---	1,2,4, 10-18, 23,25
A	WO 96 04389 A (REGENERON PHARMA) 15 February 1996 (1996-02-15) the whole document ---	
A	WO 97 23613 A (CELLTECH THERAPEUTICS LTD ;BEBBINGTON CHRISTOPHER ROBERT (GB); LAW) 3 July 1997 (1997-07-03) page 5, line 32 -page 6, line 30 page 7, line 26 - line 34; claim 26; figures 14-17 ---	
A	WO 98 02558 A (UNIV MEDICINE AND DENTISTRY OF) 22 January 1998 (1998-01-22) page 8, line 25 -page 10, line 5; figures 1,7,10,17 page 25, line 8 -page 28, line 7; claims 1,7-9 ---	
A	WO 95 10611 A (HARVARD COLLEGE) 20 April 1995 (1995-04-20) claims 5,13; example 1 ---	
A	DUSCHL A ET AL: "TRANSMEMBRANE AND INTRACELLULAR SIGNALLING BY INTERLEUKIN - 4: RECEPTOR DIMERIZATION AND BEYOND" EUROPEAN CYTOKINE NETWORK, 1996, XP002061702 figure 4 ---	
A	EP 0 864 585 A (HAYASHIBARA BIOCHEM LAB) 16 September 1998 (1998-09-16) the whole document ---	
A	WO 93 18047 A (LUDWIG INST CANCER RES) 16 September 1993 (1993-09-16) the whole document ---	

-/--

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
P,X	STAHL, N. ET AL.: "Cytokine traps: heteromeric receptor-based protein therapeutics that function as high-affinity blockers of cytokine action." FASEB JOURNAL, vol. 13, no. 7, 23 April 1999 (1999-04-23), pages A1585-Abstr.1457, XP002133324 the whole document ---	1-4, 10-14
P,X	WO 99 37772 A (BORN TERESA L ;SIMS JOHN E (US); IMMUNEX CORP (US)) 29 July 1999 (1999-07-29) page 3, line 11 - line 26 page 6, line 7 - line 9; examples 1,3,4 page 8, line 22 - line 34 page 10, line 7 - line 15 -----	1-3, 9-18,25

3

International application No
PCT/US 99/22045

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons

1 ☐ Claims Nos
because they relate to subject matter not required to be searched by this Authority, namely

2 ☐ Claims Nos
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically

3 ☐ Claims Nos
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows

see additional sheet

1 ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims

2 ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee

3 ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos

1-4,9-25 (inventions 1 (IL-2 receptor), 3 (IL-4 receptor), 4 (IL-5 receptor), 7 (IL-9 receptor), 9 (IL-13 receptor), 61 (IL-1 receptor), 65 (IL-18 receptor)

4 ☐ No required additional search fees were timely paid by the applicant Consequently, this International Search Report is restricted to the invention first mentioned in the claims, it is covered by claims Nos

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest

☒ No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 99/22045

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1: claims 1-4,10-25, all partially

Fusion protein, capable of forming a non-functional complex with a cytokine, comprising a specificity-determining component and a signal-transducing component of the IL-2 receptor, and a multimerizing component. Also nucleic acid encoding said fusion protein, (expression) vector comprising said nucleic acid, host cell transformed with said vector, and method for producing said fusion protein using said host.

Inventions 2-66: claims 1-25, all partially, as far as applicable

As invention 1, but limited to fusion proteins in which the specificity-determining component and the signal-transducing component are derived from the receptors for IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13, IL-15, GM-CSF, OSM, LIF, cardiotrophin-1, IFN-alpha, IFN-beta, IFN-gamma, B7.1/CD80, B7.2/CD86, TNF-alpha, TNF-beta, LT-beta, CD40-L, FAS-L, CD27-L, CD30-L, 4-1BBL, TGF-beta1, TGF-beta2, TGF-beta3, BMP-2, BMP-3a, BMP-3b, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8a, BMP-8b, BMP-9, BMP-10, BMP-11, BMP-15, BMP-16, EBAF, GDF-1, GDF-2, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-12, GDF-14, MIS, activin-1, activin-2, activin-3, activin-4, activin-5, IL-1, IL-10, IL-12, IL-14, IL-18, and MIF, whereby invention 2 relates to fusion protein comprising specificity-determining and signal-transducing components from the IL-3 receptor, invention 3 relates to fusion protein comprising specificity-determining and signal-transducing components from the IL-4 receptor, ..., invention 65 relates to fusion protein comprising specificity-determining and signal-transducing components from the IL-18 receptor, and invention 66 relates to fusion protein comprising specificity-determining and signal-transducing components from the MIF receptor.

For the sake of conciseness, the first subject matter is explicitly defined, the other subject matters are defined by analogy thereto.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 99/22045

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9623881 A	08-08-1996	AU 715363 B	03-02-2000
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